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In re Application of:

David A Fischoff *et al.*

Application No: 08/434,105

Filed: May 3, 1995

For: SYNTHETIC PLANT GENES AND METHOD FOR PREPARATION

Group Art Unit: 1638

Examiner: Kubelik, A.R.

PROTEST UNDER 37 CFR 1.291

ATTENTION:
DIRECTOR OF TECHNOLOGY CENTER 1600 **Fax No. (571) 273-8300**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned hereby respectfully submits the instant Protest under 37 CFR 1.291. This is the first Protest submitted in the application by the real party in interest who is submitting the Protest.

As stated in the MPEP: "A protest submitted after the mailing of a notice of allowance will not knowingly be ignored if the protest includes prior art documents which clearly anticipate or clearly render obvious one or more claims." See MPEP at 1901.04.

For the reasons that follow, it is respectfully submitted that the prior art cited herein clearly anticipates at least allowed claims 51, 59, 60, 67, 119, 120, 121, 124, 125 and 127 of U.S. application serial No. 08/434,105 ("the '105 application"). The prior art also clearly

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	08/434,105
Filing Date	May 3, 1995
First Named Inventor	Fischoff
Art Unit	1638
Examiner Name	Kubelik, A.R.
Attorney Docket Number	

ENCLOSURES *(Check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☑ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☑ Other Enclosure(s) (please identify below):

Remarks

1. Protest Under 37 CFR 1.291
2. Certificate of Service
3. Vaeck et al (1987)
4. Hofte et al (1986)
5. Beck et al (1982)

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	The Law Offices of Valerie E. Looper		
Signature			
Printed name	Valerie E. Looper		
Date		Reg. No.	33,007

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

Signature	Valerie E. Looper		
Typed or printed name	Valerie E. Looper	Date	8 January 2010

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and1.14. This collection is estimated to 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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renders obvious allowed claims 47, 49, 55, 113, 114, 115, 122, 123, 148, 149, 155, 156, 159, 160, 161 and 162.

The undersigned requests that the instant Protest be considered by the Examiner, entered into the file and that prosecution on the merits be reopened.

Proof Of Service

As required by 37 CFR 1.248, attached hereto is proof of service of the instant Protest upon the attorneys of record in the '105 application, including the date and manner of service.

Status of the Application

According to publicly available records on the USPTO Patent Application Information Retrieval (PAIR) website, U.S application serial No. 08/434,105 was filed on May 3, 1995 and is a divisional of U.S. application serial No. 07/959,506, filed on October 9, 1992, which is a File Wrapper Continuation of U.S. application serial No. 07/476,661, filed February 12, 1990, which is a continuation-in-part of U.S. application serial No. 07/315,355, filed February 24, 1989. Thus, the earliest possible effective filing date for any claim in the '105 application is February 24, 1989.

A notice of allowance was mailed in the '105 application on November 19, 2009. The issue fee is due on February 19, 2010. According to the PAIR website, as of the filing date of the instant Protest, the issue fee has not been paid in the '105 application.

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Status of the Claims

In the notice of allowance mailed November 19, 2009, claims 47, 49-51, 53-57, 59-70, 73, 77-80, 82-83, 85-86, 88-90, 93-96, 98 and 100-163 were indicated as being allowed.

Listing of Publications Relied Upon

1) Vaeck *et al.* "Transgenic plants protected by insect attack" Nature, Volume 328, July 2, 1987 (copy attached).

2) Höfte *et al.* "Structural and functional analysis of the cloned delta endotoxin of *Bacillus thuringiensis Berliner* 1715, Eur. J. Biochem., 161, 273-280 (1986) (copy attached).

3) Beck *et al.* "Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5" Gene, 19, 327-336 (1982) (copy attached).

Concise Explanation of the Relevance of the References Relied Upon

I. Claims 51, 59, 60, 67, 119, 120, 121, 124, 125 and 127 are clearly anticipated

It is respectfully submitted that at least claims 51, 59, 60, 67, 119, 120, 121, 124, 125 and 127 are clearly anticipated under 35 USC §102(b) by Vaeck *et al.* as supported by Höfte *et al.* and Beck *et al.* See the Table below, which is a claim chart correlating all the elements of a representative claim from the '105 patent with the disclosure of the Vaeck et al. reference. Höfte et al. contains a published sequence for the endotoxin in Vaeck et al., and confirms one of ordinary skill in the art at the time the '105 application was filed could determine that the deletion limitations claimed in the '105 application had been met. Beck et al. contains a

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published sequence for the neo gene used in the Vaeck et al. reference, and confirms that one of ordinary skill in the art could confirm at the time the '105 application was filed that the added sense sequence did not include the sequences to be deleted.

Vaeck *et al.* is prior art under 35 USC §102(b) because its July 2, 1987 publication date is more than one year prior to the earliest possible effective filing date (February 24, 1989) for any claim in the '105 application. Höfte *et al.* was published in 1986. Beck *et al.* was published in 1982.

Allowed claim 51 of the '105 application is representative and recites the following:

> 51. A method of making a structural gene that encodes an insecticidal protein, the method comprising:
>
> (a) starting with a portion of a coding sequence, wherein the coding sequence is derived from *Bacillus thuringiensis* (*B.t.*) and encodes an insecticidal protein and wherein the portion contains ATTTA sequences and polyadenylation signal sequences listed in Table II;
>
> (b) reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in said portion of the coding sequence by substituting sense codons for codons in said portion; and
>
> (c) making a structural gene that comprises said portion with the substitute codons and the reduced number of ATTTA and polyadenylation signal sequences, wherein the structural gene comprises a nucleotide sequence that encodes an insecticidal protein.

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Table Correlating Elements Recited in Claim 51 of the '105 Application to Express Teachings in the Prior Art Vaeck *et al.* Publication

Item	Claim 51 Elements	Express Teachings In Vaeck *et al.*
1	51. A method of making a structural gene that encodes an insecticidal protein, the method comprising	Vaeck *et al.* teach a method of making a structural gene that encodes an insecticidal protein: "Modified genes have been derived from bt2, a toxin gene cloned from one Bacillus strain. Transgenic tobacco plants expressing these genes synthesize insecticidal proteins..." Abstract. "We have used *Agrobacterium*-mediated T-DNA transfer to express chimeric *B. thuringiensis* toxin genes in tobacco plants with the objective of protecting the plants from insect attack." Page 33, column 1.
2	(a) starting with a portion of a coding sequence, wherein the coding sequence is derived from *Bacillus thuringiensis* (*B.t.*) and encodes an insecticidal protein	Vaeck *et al.* start with a portion of a coding sequence derived from *Bacillus thuringiensis* that encodes an insecticidal protein. See abstract and Figures 1a and 1b. "In plant transformation experiments, we used genes containing the entire coding sequence of bt2 as well as truncated genes."page 33, Col. 2
3	and wherein the portion contains ATTTA sequences and polyadenylation signal sequences listed in Table II;	The naturally occurring bt gene contains ATTTA sequences and polyadenylation signal sequences listed in Table II (see Figure 3 in Höfte *et al.*, the coding portion of which is reproduced herein as Exhibit B). The Bt2 coding sequence contains 28 polyadenylation signal sequences listed in Table II and 17 ATTTA sequences.

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Item	Claim 51 Elements	Express Teachings In Vaeck *et al.*
4	(b) reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in said portion of the coding sequence by substituting sense codons for codons in said portion; and	Figures 1a and 1b in Vaeck *et al.* depict the construct pGS1151. The kanamycin resistance gene, *neo*, is fused at the 3' end of the *bt2* gene fragment in pGS1151 and is referred to as *bt:neo* 23. That is, the neo sequence is substituted for a portion of the insecticidal protein coding sequence. The portion missing from the *bt2* gene contains multiple ATTTA sequences and polyadenylation signal sequences listed in Table II (See Exhibit C). Thus, the number of ATTTA sequences and the number of polyadenylation signal sequences has been reduced in the *bt:neo* 23 construct. No ATTTA sequences or polyadenylation sequences exist in the substituted region coding for kanamycin resistance (see the italicized region in Exhibit C which is the *neo* gene sequence). The *neo* gene is proven to be a sense sequence, because the transformed plants were demonstrated to express an intact NPTII protein, and also exhibit kanamycin resistance. See Vaeck et al., at page 34, Col. 2

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Item	Claim 51 Elements	Express Teachings In Vaeck *et al.*
5	(c) making a structural gene that comprises said portion with the substitute codons and the reduced number of ATTTA and polyadenylation signal sequences, wherein the structural gene comprises a nucleotide sequence that encodes an insecticidal protein.	Figure 1b in Vaeck *et al.* shows a construct with codons from the neo gene substituted for ATTTA and polyadenylation signal sequences: pGS1151 contains the *bt:neo 23* construct, wherein the neo gene is fused at the 2,173 position, so that 2 ATTTA and 9 polyadenylation signal sequences have been eliminated from the Bt sequence. The resultant sequence encodes an insecticidal protein. Bt toxin was detected in the leaves of transformed plants. A correlation was found between Bt protein and insecticidal activity in the transgenic plants. Plants transformed with the truncated *bt2* gene or the fusion constructs contained approximately ten times more Bacillus protein than those transformed with the complete *bt2* sequence. (See Vaeck et al., at para bridging pages 35 and 36, and Table 2)

As summarized above, at least claims 51, 59, 60, 67, 119, 120, 121, 124, 125 and 127 are clearly anticipated by Vaeck *et al.* as supported by Höfte *et al.* and Beck *et al.* All of the elements recited in these claims are expressly taught in Vaeck *et al.*: Höfte *et al.* and Beck *et al.* are cited as disclosures of the claimed sequences that would have been available to one of ordinary skill of the art at the time the *Vaeck et al.* reference was published.

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II. Claims 47, 49, 55, 113, 114, 115, 122, 123, 148, 149, 155, 156, 159, 160, 161 and 162 are clearly obvious

It is respectfully submitted further that at least claims 47, 49, 55, 113, 114, 115, 122, 123, 148, 149, 155, 156, 159, 160, 161 and 162 are clearly obvious under 35 USC §103(b) in view of Vaeck *et al.* combined with Höfte *et al.* and Beck *et al.* for the same reasons discussed above in the anticipation section, above.

Importantly, claim 98, which depends from claims 47, 49, 51, 59, 60, 63-64 and 67 indicates that "the insecticidal protein encoded by the structural gene comprises an amino acid sequence that is identical to the amino acid sequence of an insecticidal protein from *B.t.*, or an insecticidal fragment thereof." By the doctrine of claim differentiation, claims 47, 49, 51, 59, 60, 63-64 and 67 are necessarily broader in scope than claim 98 and would encompass insecticidal proteins comprising an amino acid sequence that is not identical to the amino acid sequence of an insecticidal protein from *B.t.*, or an insecticidal fragment thereof.

CONCLUSION

The undersigned respectfully requests that the instant Protest be considered by the Examiner, entered into the file and that prosecution on the merits be reopened.

Respectfully submitted,

Date: January 8, 2010

By: Valerie E. Looper
Valerie E. Looper
Registration No. 33,007

The Law Offices of Valerie E. Looper
11726 Lightfall Ct.
Columbia, MD 21044

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Exhibit A

Table II from '105 Application

TABLE II

List of Sequences of the Potential Polyadenylation Signals	
AATAAA*	AAGCAT
AATAAT*	ATTAAT
AACCAA	ATACAT
ATATAA	AAAATA
AATCAA	ATTAAA**
ATACTA	AATTAA**
ATAAAA	AATACA**
ATGAAA	CATAAA**

*indicates a potential major plant polyadenylation site.
**indicates a potential minor animal polyadenylation site.

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Exhibit B

Coding Sequence of delta endotoxin of *Bacillus thuringiensis Berliner 1715*. See Figure 3 in Höfte *et al.* "Structural and functional analysis of the cloned delta endotoxin of *Bacillus thuringiensis Berliner* 1715, Eur. J. Biochem., 161, 273-280 (1986).

```
   1 atggataaca atccgaacat caatgaatgc attccttata attgtttaag taaccctgaa
  61 gtagaagtat taggtggaga aagaatagaa actggttaca ccccaatcga tatttccttg
 121 tcgctaacgc aatttctttt gagtgaattt gttcccggtg ctggatttgt gttagcacta
 181 gttgatataa tatggggaat ttttggtccc tctcaatggg acgcatttct tgtacaaatt
 241 gaacagttaa ttaaccaaag aatagaagaa ttcgctagga accaagccat ttctacatta
 301 gaaggactaa gcaatcttta tcaaatttac gcagaatctt ttagacagtg ggaagcagat
 361 cctactaatc cagcattaag agaagagatg cgtattcaat tcaatgacat gaacactgcc
 421 cttacaaccg ctattcctct ttttgcagtt caaaattatc aagttcctct tttatcagta
 481 tatgttcaag ctgcaaattt acatttatca gttttgagag atgtttcagt gtttgcacaa
 541 aggtggggat ttgatgccgc gactatcaat agtcgttata atgatttaac taggcttatt
 601 ggcaactata cagatcatgc tgtacgctgg tacaatacgg gattacagcg tgtatgggga
 661 ccggattcta gagattggat aagatataat caatttagaa gaggaattaac actaactgta
 721 ttagatatcg tttctctatt tccgaactat gatagtagaa cgtatccaat tcgaacagtt
 781 tcccaattaa caagagaaat ttatacaaac ccagtattag aaaattttga tggtagtttt
 841 cgaggctcgg ctcagggcat agaaggaagt attaggagtc cacatttgat ggatatactt
 901 aacagtataa ccatctatac ggatgctcat agaggagaat attattggtc agggcatcaa
 961 ataatggctt ctcctgtagg gttttcgggg ccagaattca cttttccgct atatggaact
1021 atgggaaatg cagctccaca acaacgtatt gttgctcaac taggtcaggg cgtgtataga
1081 acattatcgt ccactttata tagaagacct tttaatatag ggataaataa tcaacaacta
1141 tctgttcttg acgggacaga atttgcttat ggaacctcct caaatttgcc atccgctgta
1201 tacagaaaaa gcggaacggt agattcgctg gatgaaatac cgccacagaa taacaacgtg
1261 ccacctaggc aaggatttag tcatcgatta agccatgttt caatgtttcg ttcaggcttt
1321 agtaatagta gtgtaagtat aataagagct cctatgttct cttggataca tcgtagtgct
1381 gaatttaata atataattcc ttcatcacaa attacacaaa taccttttaac aaaatctact
1441 aatcttggct ctggaacttc tgtcgttaaa ggaccaggat ttacaggagg agatattctt
1501 cgaagaactt cacctggcca gatttcaacc ttaagagtaa atattactgc accattatca
1561 caaagatatc gggtaagaat tcgctacgct tctaccadaa atttacaatt ccatacatca
1621 attgacggaa gacctattaa tcagggaat ttttcagcaa ctatgagtag tgggagtaat
1681 ttacagtccg gaagctttag gactgtaggt tttactactc cgtttaactt ttcaaatgga
1741 tcaagtgtat ttacgttaag tgctcatgtc ttcaattcag gcaatgaagt ttatatagat
1801 cgaattgaat ttgttccggc agaagtaacc tttgaggcag aatatgattt agaaagagca
1861 caaaaggcgg tgaatgagct gtttacttct tccaatcaaa tcgggttaaa aacagatgtg
1921 acggattatc atattgatca agtatccaat ttagttgagt gtttatctga tgaattttgt
1981 ctggatgaaa aaaaagaatt gtccgagaaa gtcaaacatg cgaagcgact tagtgatgag
2041 cggaatttac ttcaagatcc aaactttaga gggatcaata gacaactaga ccgtggctgg
2101 agaggaagta cggatattac catccaagga ggcgatgacg tattcaaaga gaattacgtt
2161 acgctattgg gtacctttga tgagtgctac ttaacgtatt tatatcaaaa aatagatgag
2221 tcgaaattaa aagcctatac ccgttaccaa ttaagagggt atatcgaaga tagtcaagac
2281 ttagaaatct atttaattcg ctacaatgcc aaacacgaaa cagtaaatgt gccaggtacg
2341 ggttccttat ggcgcctttc agccccaagt ccaatcggaa aatgtgccca tcattcccat
2401 catttctcct tggacattga tgttggatgt acagacttaa atgaggactt aggtgtatgg
2461 gtgatattca agattaagac gcaagatggc catgcaagac taggaaatct agaatttctc
2521 gaagagaaac cattagtagg agaagcacta gctcgtgtga aaagagcgga gaaaaaatgg
2581 agagacaaac gtgaaaaatt ggaatgggaa acaaatattg tttataaaga ggcaaaagaa
2641 tctgtagatg ctttatttgt aaactctcaa tatgatagat tacaagcgga taccaacatc
2701 gcgatgattc atgcggcaga taaacgcgtt catagcattc gagaagctta tctgcctgag
2761 ctgtctgtga ttccgggtgt caatgcggct atttttgaag aattagaagg gcgtattttc
2821 actgcattct ccctatatga tgcgagaaat gtcattaaaa atggtgattt taataatggc
2881 ttatcctgct ggaacgtgaa agggcatgta gatgtagaag aacaaaacaa ccaccgttcg
2941 gtccttgttg ttccggaatg ggaagcagaa gtgtcacaag aagttcgtgt ctgtccgggt
3001 cgtggctata tccttcgtgt cacagcgtac aaggagggat atggagaagg ttgcgtaacc
3061 attcatgaga tcgagaacaa tacagacgaa ctgaagttta gcaactgtgt agaagaggaa
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```
3121 gtatatccaa acaacacggt aacgtgtaat gattatactg cgactcaaga agaatatgag
3181 ggtacgtaca cttctcgtaa tcgaggatat gacggagcct atgaaagcaa ttcttctgta
3241 ccagctgatt atgcatcagc ctatgaagaa aaagcatata cagatggacg aagacacaat
3301 ccttgtgaat ctaacagagg atatggggat tacacaccac taccagctgg ctatgtgaca
3361 aaagaattag agtacttccc agaaaccgat aaggtatgga ttgagatcgg agaaacggaa
3421 ggaacattca tcgtggacag cgtggaatta cttcttatgg aggaataa
```

The sequence shown above is the native Bt2 coding sequence (full length). The Bt2 gene coding sequence was obtained from Bt berliner 1715 (Genbank accession X04698), which corresponds to the Höfte *et al.* 1986 (Eur. J. Biochem. 161, 273-280) publication of the Bt2 gene sequence.

The polyadenylation signal sequences of Table II (bold single underlining) and the ATTTA sequences (bold double underlining), as claimed in the allowed claims of US application 08/434,105, are indicated. The location of the end of the Bt2 sequence, position 2,173, is indicated in bold italic caps and underlined. This is the location of the Bt2-nptII fusion (*bt:neo* 23) described in Vaeck *et al.*

Hence, there are a total of 28 polyadenylation signal sequences as listed in Table II of the '105 application (note that some of these sequences overlap with each other) and 17 ATTTA sequences in the native Bt2 coding sequence.

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Exhibit C

Sequence of *bt:neo* 23 fusion gene construct shown in Figure 1b of Vaeck *et al.*

```
   1 atggataaca atccgaacat caatgaatgc attcctata attgtttaag taaccctgaa
  61 gtagaagtat taggtggaga aagaatagaa actgcttaca ccccaatcga tatttccttg
 121 tcgctaacgc aatttctttt gagtgaattt gttcccggtg ctggatttgt gttacgacta
 181 gttgatataa tatggggaat ttttggtccc tctcaatggg acgcatttct tgtacaaatt
 241 gaacagttaa ttaaccaaag aatagaagaa ttcgctaggg accaagccat ttctagatta
 301 gaaggactaa gcaatcttta tcaaatttac gcagaatctt ttagagagtg ggaacgagat
 361 cctactaatc cagcattaag agaagagatg cgtattcaat tcaatgacat gaacagtgcc
 421 cttacaaccg ctattcctct ttttgcagtt caaaattatc aagttcctct tttatcagta
 481 tatgttcaag ctgcaaattt acatttatca gttttgagag atgtttcagt gtttcgacaa
 541 aggtggggat ttgatgccgc gactatcaat agtccttata atgatttaac taggcttatt
 601 ggcaactata cagatcatgc tgtacgctgg tacaatacgg gattagagcg tgtatgggga
 661 ccggattcta gagattggat aagatatatt caatttagaa gagaattaac actaactgta
 721 ttagatatcg tttctctatt tccgaactat gatagtagaa cgtatccaat tcgaacagtt
 781 tcccaattaa caagagaaat ttatacaaac ccagtattag aaaattttga tggtagtttt
 841 cgaggctcgg ctcagggcat agaaggaagt attaggagtc cacatttgat ggatatactt
 901 aacagtataa ccatctatac ggatgctcat agaggagaat attattggtc agggcatcaa
 961 ataatggctt ctcctgtagg gttttcgggg ccagaattca cttttccgct atatggaact
1021 atgggaaatg cagctccaca acaacgtatt gttgctcaac taggtcaggg cgtgtataga
1081 acattatcgt ccactttata tagaagacct tttaatatag ggataaatta tcaacaacta
1141 tctgttcttg acgggacaga atttgcttat ggaacctcct caaatttgcc atccgctgta
1201 tacagaaaaa gcggaacggt agattcgctg gatgaaatac cgccacagaa taacaacgtg
1261 ccacctaggc aaggatttag tcatcgatta agccatgttt caatgtttcg ttcaggcttt
1321 agtaatagta gtgtaagtat aataagagct cctatgttct cttggataca tcgtagtgct
1381 gaatttaata tataattccc ttcatcacaa attacacaaa taccttaac aaaatctact
1441 aatcttggct ctggaacttc tgtcgttaaa ggaccaggat ttacaggagg agatattctt
1501 cgaagaactt cacctggcca gatttcaacc ttaagagtaa atattactgc accattatca
1561 caaagatatc gggtaagaat tcgctacgct tctaccacaa atttacaatt ccatacatca
1621 attgacggaa gacctattaa tcagggaat ttttcagcaa ctatgagtag tgggagtaat
1681 ttacagtccg gaagctttag gactgtaggt tttactactc cgtttaactt ttcaaatgga
1741 tcaagtgtat tacgttaag tgctcatgtc ttcaattcag gcaatgaagt ttatatagat
1801 cgaattgaat ttgttccggc agaagtaacc tttgaggcag aatatgattt agaaagagca
1861 caaaaggcgg tgaatgagct gtttacttct tccaatcaaa tcgggttaaa aacagatgtg
1921 acggattatc atattgatca agtatccaat tagttgagt gtttatctga tgaattttgt
1981 ctggatgaaa aaaaagaatt gtccgagaaa gtcaaacatg cgaagcgact tagtgatgag
2041 cggaatttac ttcaagatcc aaactttaga gggatcaata gacaactaga ccgtggctgg
2101 agaggaagta cggatattac catccaagga ggcgatgacg tattcaaaga gaattacgtt
2161 acgctattgg gtaccgatcc gccaagcttg gatggattgc acgcaggttc tccggccgct
2221 tgggtggaga ggctattcgg ctatgactgg gcacaacaga caatcggctg ctctgatgcc
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2461 ggcgaagtgc cggggcagga tctcctgtca tctcaccttg ctcctgccga gaaagtatcc
2521 atcatggctg atgcaatgcg gcggctgcat acgcttgatc cggctacctg cccattcgac
2581 caccaagcga aacatcgcat cgagcgagca cgtactcgga tggaagccgg tcttgtcgat
2641 caggatgatc tggacgaaga gcatcagggg ctcgcgccag ccgaactgtt cgccaggctc
2701 aaggcgcgca tgcccgacgg cgaggatctc gtcgtgaccc atggcgatgc ctgcttgccg
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2881 gaatgggctg accgcttcct cgtgctttac ggtatcgccg ctcccgattc gcagcgcatc
2941 gccttctatc gccttcttga cgagttcttc tga
```

Nucleotides 1-2173 depicted in Exhibit C are part of the *bt:neo* 23 construct. Indicated with double underlining and bold italics is a 16bp linker sequence; the nucleotides 3' (downstream) from this linker in italics and underlined are the nptII nucleotides taken from Beck *et al.* which

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encode *neo*, the kanamycin resistance gene, starting at position 13 (1982, Genbank accession V00618.1). There are no ATTTA or polyadenylation signal sequences in this region.

The DNA shown here is the truncated Bt2-nptII fusion protein gene named *bt:neo* 23 of Ti plasmid pGS1151 illustrated in Figure 1 in Vaeck *et al.* (1987) or EP0193259. It is a gene fusion between the Bt2 and nptII sequences as shown in Fig. 24 of EP0193259.

The polyadenlyation signal sequences of Table II as claimed in the allowed claims of US application 08/434,105 are highlighted (bold single underline), as well as ATTTA sequences as claimed (bold double underline).

Hence, when compared with Exhibit B, 2 ATTTA and 9 polyadenylation signal sequences as claimed were removed/deleted by changing the codons of the native Bt2 sequence for codons of the nptII gene (which does not contain any polyadenylation signal sequences or ATTTA sequences as claimed).

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ARTICLES

Transgenic plants protected from insect attack

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The Gram-positive bacterium Bacillus thuringiensis *produces proteins which are specifically toxic to a variety of insect species. Modified genes have been derived from* bt2, *a toxin gene cloned from one* Bacillus *strain. Transgenic tobacco plants expressing these genes synthesize insecticidal proteins which protect them from feeding damage by larvae of the tobacco hornworm.*

MODERN agriculture uses a wide variety of insecticides to control insect damage. Most of them are chemically synthesized. Notable exceptions are the insect toxins produced by *Bacillus thuringiensis*: spore preparations of this Gram-positive bacterium have been used for more than 20 years as a biological insecticide[1]. The insecticidal activity resides in crystalline inclusion bodies produced during sporulation of the bacteria, which are composed of proteins (termed delta endotoxins) specifically toxic against a variety of insects. Different strains of *B. thuringiensis* differ in their spectra of insecticidal activity. Most are active against Lepidoptera, but some strains specific to Diptera[2,3] and Coleoptera[4,5] have been identified. The crystals dissolve in the alkaline conditions of the insect midgut and release proteins of relative molecular mass 65,000–160,000 (M_r 65K–160K)[2,5,6] which are proteolytically processed by midgut proteases to yield smaller toxic fragments[7]. *B. thuringiensis* insect toxins are highly specific, in that they are not toxic to other organisms. Hence, they are safe insecticides and present an interesting alternative to chemical control agents. Their commercial use however is limited by high production costs and the instability of the crystal proteins when exposed in the field.

We have used *Agrobacterium*-mediated T-DNA transfer[8] to express chimaeric *B. thuringiensis* toxin genes in tobacco plants with the objective of protecting the plants from insect attack. We show here that a defence mechanism against phytophagous insects can be devised by genetic engineering.

Modified *Bacillus* toxins

We have reported[9] the cloning of the *bt2* gene from *B. thuringiensis* strain berliner 1715 and the characterization of the recombinant polypeptide expressed in *Escherichia coli*. This protein, termed Bt2, is 1,155 amino acids long and is a potent toxin to several lepidopteran larvae, such as those of *Manducta sexta*, a pest of tobacco. Bt2 is a protoxin and generates a smaller polypeptide of M_r 60K which retains full toxic activity[9]. The smallest fragment of Bt2 that is still fully toxic has been mapped in the NH_2-terminal half of the protein, between amino-acid positions 29 and 607 (ref. 9) (and see Fig. 1*a*).

In plant transformation experiments, we used chimaeric genes containing the entire coding sequence of *bt2* as well as truncated genes. A diagram of the chimaeric genes is shown in Fig. 1*b*. Some of our T-DNA constructs include a chimaeric neomycin phosphotransferase gene (*neo*) as a marker selectable in plants[10]. Others carry translational fusions between fragments of *bt2* and the *neo* gene. Fusions to the 5′ end of the *neo* gene still confer kanamycin resistance in bacteria[11] and in plants[12]. Plasmid pLB884 (ref. 9) contains the truncated gene *bt884* and encodes an NH_2-terminal fragment of Bt2 up to amino-acid position 610. In *E. coli* it produces a polypeptide of the expected size which is fully toxic towards insect larvae[9].

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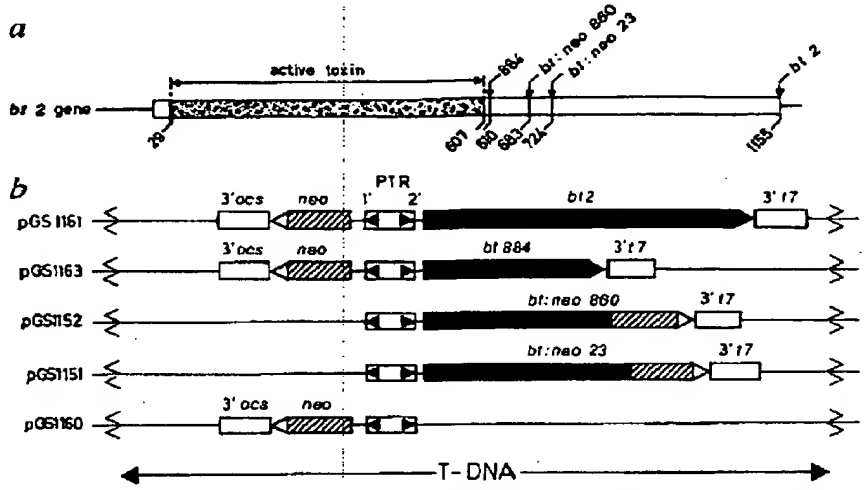

Fig. 1 *a*, Structure of the *bt2* gene. The smallest gene fragment encoding an active insect toxin is indicated, and the 3' end positions (codon numbers) of the different truncated genes. *b*, Chimaeric genes derived from *bt2* present in plant expression vectors. The 5' end of the *bt2* coding sequence is fused to the 2' promoter fragment of the TR DNA. pGS 1161 contains the intact *bt2* gene. The *bt2* segment in pGS1163 ends at nucleotide position 1,830 of the *bt2* coding sequence. In pGS1151 and pGS1152, the *neo* gene has been fused to 5' fragments of the *bt2* gene at positions 2,173 and 2,050 of the *bt2* coding sequence, respectively. PTR, a 482-base pair (bp) fragment containing the TR DNA 1' and 2' promoters, isolated from pOP443 (ref. 15); 3'tr7, a 211-bp fragment containing the polyadenylation site of the TL DNA gene 7 (ref. 23); 3'ocs, a 706-bp *Pou*II fragment containing the polyadenylation site of the octopine synthase gene[24]. Plasmids pGS1160, pGS1161 and pGS1163 were made using the intermediate plasmid pGSH160 which contains a chimaeric *neo* gene[14]. For pGS1151 and pGS1152, containing the *bt2*:*neo* fusion genes pGSH150, a derivative of pGSH160, lacking the *neo* gene and 3' ocs, was used.

Plasmids pLBKm860 and pLBKm23 (H.H. *et al.*, in preparation) contain fusion genes *bt*:*neo860* and *bt*:*neo23* encoding NH_2-terminal fragments of Bt2 up to amino-acid position 683 and 724, respectively. *E. coli* cells harbouring these plasmids are resistant to kanamycin and produce fusion proteins Bt:NPT860 and Bt:NPT23 which have the expected M_r of 106K and 110K respectively and which react with anti-Bt2 (Fig. 2A) and anti-NPTII antibodies. At least 50% of the fusion proteins is present in a soluble form in the bacterial cells. The neomycin phosphotransferase activity of the fusion proteins, as determined in an *in situ* assay[13], is comparable to wild-type NPTII activity. Little or no enzymatic activity was exhibited by polypeptides of lower M_r (Fig. 2*b*). We conclude that the fusion proteins are relatively stable and responsible for the kanamycin-resistant phenotype. Insect assays revealed that, on a molar basis, the fusion proteins exhibit the same toxicity towards *M. sexta* larvae as intact Bt2 protein which has an LD_{50} value on first instar larvae of 4 ± 2 ng per larva.

Plant expression vectors

The intact and modified *B. thuringiensis* toxin genes were inserted between the T-DNA borders of plant expression vector pGSH160 (for *bt2* and *bt884*) or pGSH150 (ref. 14) (for *bt*:*neo860* and *bt*:*neo23*). These plasmids contain the promoter of the 2' gene, a constitutive promoter which directs expression of mannopine synthase in the TR DNA of plasmid pTiA6 (ref. 15). The *Bacillus* genes are followed by a termination signal provided by the 3' end of gene 7 of pTiA6. The resulting plasmids were mobilized into the *Agrobacterium* recipient C58C1 Rif^R pGV2260. The latter contains an octopine Ti plasmid from which the whole T-DNA region has been deleted and replaced by pBR322 (ref. 14). Recombination between pGV2260 and the expression vector through the homologous pBR322 sequences produced Ti plasmids pGS1161, pGS1163, pGS1151 and pGS1152 containing *bt2*, *bt884*, *bt*:*neo23* and *bt*:*neo860*, respectively (Fig. 1).

Two approaches were used to increase the probability of obtaining high levels of toxin expression in plants. First, the expression levels directed from the 1' and 2' promoters from the TR DNA were found to be coordinated[15]. Consequently we expected that expression of the *neo* gene controlled by the 1' promoter in plants transformed with pGS1161 and pGS1163, would be correlated with transcription of the toxin gene. Second, we anticipated that plant cells transformed with pGS1151 or pGS1152, containing *bt*:*neo* fusions, would produce fusion proteins expressing NPTII activity. Selection for high levels of kanamycin resistance would allow us to select directly for transformed clones producing substantial amounts of *B. thuringiensis* protein.

Transformation of tobacco plants

Transgenic tobacco plants were obtained by leaf disk infection[14,16] of *Nicotiana tabacum* var. Petit Havana SR1 (ref. 17). Shoots resistant to 50, 100 or 200 $\mu g\ ml^{-1}$ kanamycin were selected in all transformation experiments, indicating that the fusion genes indeed confer NPTII activity on transformed plant cells. Individual transformed plants were grown up and subsequently assayed for kanamycin resistance by testing their ability to produce callus from leaf disks on increasing concentrations of kanamycin and the more toxic aminoglycoside antibiotic G418. Most of the transgenic plants expressing an intact NPTII protein produced highly resistant calli, growing on 1,000 $\mu g\ ml^{-1}$ kanamycin and on 100 $\mu g\ ml^{-1}$ G418. In contrast, plants that expressed a Bt:NPTII fusion protein readily fell into different

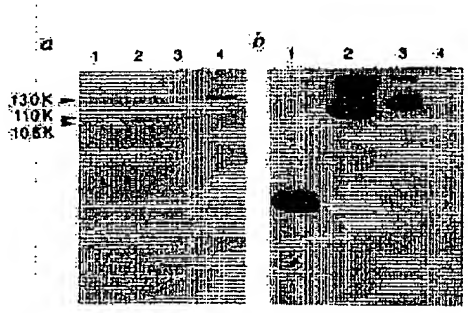

Fig. 2 Antigenic properties and enzymatic activity of Bt:NPTII fusion proteins. *a*, Western blot analysis[22] of crude extracts of *E. coli* clones producing intact NPTII enzyme (lane 1), Bt:NPT860 (lane 2) or Bt:NPT23 fusion protein (lane 3). Lane 4, purified Bt2. Blots were incubated with a diluted anti-Bt2 serum and subsequently with alkaline phosphatase-labelled anti-rabbit immunoglobulin. Substrates were 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt and *p*-nitro-blue tetrazolium chloride (Sigma). *b*, Detection of NPTII activity in samples identical to those in *a*, by *in situ* phosphorylation of kanamycin with ^{32}P-ATP (ref. 13). All samples in *a* and *b* contain 0.25 μg protein per lane.

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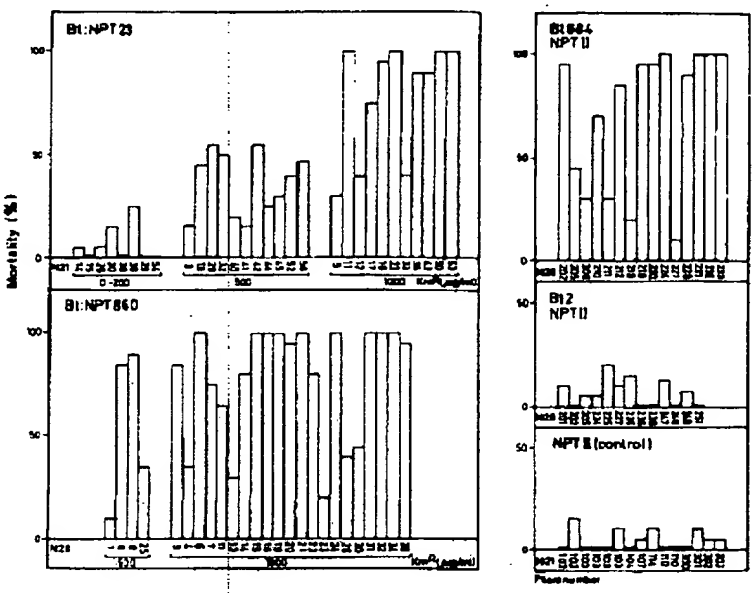

Fig. 3 Insect toxicity of transgenic tobacco plants expressing *B. thuringiensis* protein. Mortality of *Manduca sexta* larvae after 6 days of feeding on tobacco leaves is shown. The serial number of each plant is given below each column, and in the left-hand panels plants are grouped according to the concentration (in $\mu g\ ml^{-1}$) of kanamycin to which they are resistant. Each panel is labelled with the protein expressed (see Fig. 1). Plants were tested 4–6 weeks after transfer to the greenhouse, when they were about 40-cm high and had between six and eight full grown leaves. Two leaf disks of 4 cm in diameter were placed on wet filter paper in Petri dishes and infested with two batches of 10 first instar larvae of *M. sexta*. Leaf disks were replaced daily. Tests were conducted in a growth chamber at 25 °C, 75% relative humidity and under a 16 h light/8 h dark cycle.

Table 1 Kanamycin and G418 resistance in transgenic tobacco plants

Agrobacterium strain	Expression products of chimaeric genes	No. of plants resistant: Kanamycin ($\mu g\ ml^{-1}$) <50	50	100	200	500	1000	G418 ($\mu g\ ml^{-1}$) <10	10	100	Total
pGS1151	Bt: NPT23	3	1	7	17	14	10	51	1	0	52
pGS1152	Bt: NPT860	0	0	0	0	5	31	35	1	0	36
pGS1161	Bt2, NPTII	0	0	0	0	0	14	0	0	14	14
pGS1163	Bt884, NPTII	0	0	0	0	1	14	1	0	14	15
pGS1160	NPTII	0	0	0	0	2	12	2	0	12	14

Plants were scored according to the highest concentration of antibiotic on which callus could be induced from leaf disks from *in vitro* grown plants[21].

classes of kanamycin resistance (Table 1). Because the specific enzymatic activity of the fusion proteins is comparable to that of intact NPTII, we presume that these fusion proteins are present in lower amounts in the plant cells than intact NPTII protein.

Insecticidal activity in transgenic plants

Leaves of transgenic plants containing the four types of *B. thuringiensis* gene constructs were fed to *M. sexta* larvae in order to evaluate whether the levels of toxin in the plants would be insecticidal. Mortality rates of *M. sexta* larvae were monitored after 6 days of feeding on leaves of transformed plants (Fig. 3). We found that high toxicity to insects, resulting in 75–100% mortality of the larvae, was observed in about one quarter of the plants that expressed the longer fusion protein Bt: NPT23 and in about two-thirds of those with the shorter Bt: NPT860. Thus, the Bt: NPTII fusions allowed us to select transformants that express levels of the toxin sufficiently high to be insecticidal. Second, insect toxicity caused by the fusion proteins is directly correlated with the level of kanamycin resistance of the transformed plant. In addition, the short fusion generates a larger fraction of transformants expressing high kanamycin resistance and insect toxicity than the longer fusion, suggesting that the shorter *bt: neo860* gene provides higher levels of biologically active protein than the longer *bt: neo23*. Clear insecticidal activity was also detected in most of the 15 plants expressing the truncated *bt884* gene, of which two-thirds induced more than 75% larval death. None of the plants transformed with the full length *bt2* gene produced insect killing activity above levels obtained in NPTII-expressing control plants. These experiments indicate that for the promoter gene constructs we used, only truncated *bt2* genes give rise to expression levels that are strongly insecticidal in transgenic tobacco.

Protection from insect damage

To test whether expression of modified *bt2* genes in plants results in effective protection against insect damage, selected transgenic plants were grown in the greenhouse and were infested with freshly hatched larvae of *M. sexta*. Plants were kept under conditions that were optimal for survival and growth of the insects. Plants N21-11 and N28-16 were highly protected, because the larvae stopped feeding within 18 hours and all were killed within three days. The damage caused by single larvae was limited to areas of only a few square millimetres (Fig. 4). Other plants, such as N21–53 or N28-6, suffered slightly more damage. On these plants however all the larvae were killed after six days. Control plants such as N21–110, transformed with pGS1160, or untransformed SR1, were severely damaged within 4–6 days and were entirely consumed after 12 days.

Expression of chimaeric genes in plants

Quantitative detection of *B. thuringiensis* toxin in the leaves of transformed plants was performed using a sensitive ELISA, with a mixture of monoclonal antibodies specific for the NH_2-terminal region of the protein. A correlation was found between the quantity of *Bacillus* protein and insecticidal activity in the transgenic plants. Plants transformed with the truncated *bt2* gene or the fusion constructs contain approximately ten times

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more *Bacillus* protein than those transformed with the complete *bt2* sequence (Table 2). Thus, the failure to obtain insect-resistant plants using the intact *bt2* gene is most probably due to inefficient protein synthesis in these transformed plant cells.

Transgenic plants that express the shorter Bt : NPT860 protein are on average more effective in killing insects and express higher levels of toxin than those expressing the longer Bt : NPT23 protein (Table 2). The Bt : NPTII fusion proteins detected in leaf extracts of the transformed plants had the expected size, as determined in Western blots. NPTII enzyme activity was exclusively associated with these fusion proteins as determined by *in situ* NPTII assays. Production of incomplete proteins or degradation in the plant cells was not observed. Thus, transformed tobacco plants produced fully functional fusion proteins of the size of the intact gene product.

The steady-state *Bacillus* messenger RNA levels in the transgenic plants were low and could not be reliably detected in Northern blot analysis. Therefore, they were quantified using ribonuclease protection experiments[18]. A probe containing the 5′ fragment of the *bt2* coding sequence up to nucleotide position 186 was synthesized using the SP6 transcription system and annealed to total RNA from the leaves of transgenic plants. After ribonuclease digestion, the protected fragments were run on a denaturing polyacrylamide gel. The results showed that RNA levels in the leaves of the transformed plants were correlated with *B. thuringiensis* protein levels (data not shown). The *B. thuringiensis* mRNA in N28-16, the plant that produces the highest level of protein, corresponds to ~0.0001% of the poly(A)$^+$ mRNA. The *Bacillus* protein detected in this plant represents 0.02% of the total soluble protein, or 3 μg of this protein per gram fresh leaf tissue. A fivefold lower level, as present in plant N28-34, was sufficient to induce 100% killing in a 6-day insect assay (Table 2). For comparison, the expression levels of the intact *neo* gene also driven by the TR 2′ promoter, varied between 10 and 40 μg of NPTII per gram leaf tissue (0.07–0.27% of total protein). This difference in amount of protein is consistent with the lower kanamycin resistance levels of plants transformed with the *bt : neo* fusions compared to those transformed with the intact *neo* gene (Table 1).

Inheritance of the protection

Eighty-five transgenic tobacco plants, transformed with the four types of chimaeric *B. thuringiensis* genes and expressing various levels of active insect toxin, were grown in the greenhouse. All grew normally and were indistinguishable from controls in morphology and vigour of growth. We analysed the inheritance of the kanamycin resistance in eleven plants expressing *bt : neo* genes. Most of these plants contained one (N21-23, N21-50, N28-31, N28-34, N28-21) or two (N21-35, N28-19, N28-24, N28-32) kanamycin resistance loci, as confirmed by Southern blot analysis. Interestingly, some plants that were recognized as producing a large amount of *Bacillus* protein, such as N21-11 and N28-16, generated exclusively kanamycin-resistant F_1 seedlings. DNA analysis showed the presence of at least five copies of the T-DNA in these plants.

F_1 progeny from some transgenic plants were assayed for the expression of insecticidal activity. Insect toxicity was correlated with the kanamycin resistance marker in the F_1 progeny of plants N21-23, N21-50 and N28-34. Insecticidal activity was similar to that observed in the parental plants. Approximately 15 F_1 progeny of N21-11 and N28-16 were analysed. They all induced 100% killing of *M. sexta* larvae in the standard 6-day assay. Toxin levels in the F_1 progeny of N21-11 varied between 20 and 50 ng per mg of total protein, comparable to the 30 ng per mg in the parental plant.

Discussion

Four chimaeric genes containing modified *Bacillus* toxin genes under the control of the 2′ promoter of the *Agrobacterium* TR DNA, have been transferred into tobacco plants. All contain the toxic core of the Bt2 protein; *bt2* encodes the complete M_r 130K protoxin, *bt884* is a 5′ fragment of *bt2* up to codon 610. *Bt : neo23* and *bt : neo860* encode fusion proteins which are relatively stable, both in bacteria and plants, and which retain full insect toxicity and NPTII enzyme activity.

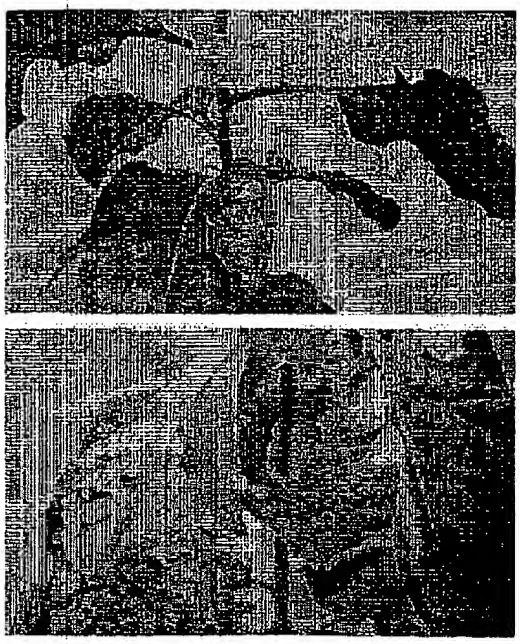

Fig. 4 Protection from insect feeding damage in transgenic plants expressing *B. thuringiensis* protein. Plants 40–50-cm high were infested in the greenhouse with fifteen *M. sexta* larvae per plant. *a*, On N21-11, expressing Bt : NPT23, all larvae died within three days. Leaf damage is very limited. *b*, Damage on a control plant expressing NPTII. Pictures were taken after 11 days.

Insecticidal levels of toxin were produced when truncated *Bacillus* genes or fusion constructs were expressed in transgenic plants. Mortality rates among *M. sexta* larvae feeding on transgenic leaf material depended on the amount of toxic polypeptide produced. Typically, greenhouse grown plants producing more than 0.004% of their protein as the toxin produced 100% mortality in 6-day feeding assays. Some of the plants we have selected, contain toxin at three to five times this level (N21-11 and N28-16). In greenhouse tests, these plants were well protected from leaf damage caused by insects. Quantification also showed that the toxin expressed in plants has the same specific activity as in a bacterial host.

No significant insecticidal activity could be obtained using the intact *bt2* coding sequence, despite the fact that the same promoter was used to direct its expression. Intact Bt2 protein and RNA amounts in the transgenic plant leaves were 10–50 times lower than those for the truncated *B. thuringiensis* polypeptide or the fusion proteins. Expression levels were not significantly influenced by fusing the *neo* gene to the *bt2* sequence, but rather by the length of the *bt2* fragment. Why the complete *bt2* gene is not expressed at an equally high level in plant cells, is not known. Several parameters, such as differential RNA stability and translation efficiency might be important.

We observed in transgenic plants containing the *bt : neo* fusion constructs a correlation between insecticidal activity and resistance to kanamycin. Three-quarters of all plants resistant to 1,000 μg ml^{-1} kanamycin induced 75–100% insect mortality. Such fusion genes can be used to select efficiently for transformed plants expressing strong insecticidal activity through direct selection for high kanamycin resistance.

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Table 2 Insecticidal activity and *B. thuringiensis* protein content of transformed plants

Toxic protein	Plant	*Bacillus* protein detected (ng per mg total protein)	Insecticidal effect (% mortality) Day 3	Day 4	Day 6	Weight reduction in surviving larvae (%)
Bt: NPT23	N21-11	33 (132)	90	100	100	—
	N21-35	6.9 (71)	0	40	90	57
	N21-17	2.6	0	15	75	71
	N21-18	5.7	25	50	90	80
	N21-32	2.5	5	40	50	39
	N21-41	4.3	0	0	15	34
	N21-43	4.7	10	40	80	66
Bt: NPT860	N28-16	42 (190)	100	100	100	—
	N28-34	6.9 (42)	70	85	100	—
	N28-6	13	80	90	100	—
	N28-15	10	75	95	100	—
	N28-19	6.2	35	65	100	—
	N28-21	7.0	45	75	100	—
	N28-24	12	60	90	100	—
	N28-31	6.3	55	80	100	—
	N28-32	14	85	90	100	—
Bt2	N21-105	1.3 (5.5)	0	5	15	7
	N21-225	1.2 (2.1)	5	10	20	17
	N21-201	<1	0	5	10	23
	N21-236	1.3	0	0	0	0
	N21-238	<1	0	0	0	0
	N21-249	1.8	0	10	10	10
Bt884	N28-212	30 (125)	100	100	100	—
	N28-220	11 (40)	60	95	100	—
	N28-219	11	55	65	95	85

Values in parentheses refer to amounts detected in greenhouse grown plants. Extracts were prepared from leaves of propagated *in vitro* plants or from leaves of plants grown in a greenhouse that had between six and eight fully expanded leaves. Leaf tissue was ground up and subsequently sonicated (10 s at 50 W) in extraction buffer (Na_2CO_3, 50 mM at pH10; dithiothreitol (DTT), 5 mM; leupeptin, 1 mg ml^{-1}; Triton X-100, 0.05%; EDTA 50 mM; phenylmethylsulphonyl fluoride (PMSF), 0.19 mg ml^{-1}). The extract was cleared by centrifugation and *B. thuringiensis* polypeptides in the supernatant were quantified using an indirect enzyme-linked immunosorbent assay[22] (ELISA). Polyvinyl microtitre plates were coated with a goat antibody against *B. thuringiensis* crystal protein. Plant extract dilutions were incubated at 4 °C for 2 h in the coated wells. After rinsing, bound antigen was reacted with a mixture of four distinct monoclonal antibodies against Bt2 and subsequently with an alkaline phosphatase conjugated goat anti-mouse immunoglobulin antibody. The bound enzyme conjugate was detected by adding *p*-nitrophenyl phosphate as a substrate, and relative quantities were determined by measuring absorbance values at 405 nm. The monoclonal antibodies used specify antigenic epitopes located between amino-acid positions 29 and 222 in the NH_2-terminal region of the Bt2 protein (H. Vanderbruggen *et al.*, in preparation). To quantify the *Bacillus* protein levels in transgenic plants, ELISA binding curves of leaf extracts were compared to standard binding curves, obtained by diluting known quantities of the purified homologous protein in control extracts from non-transformed SR1 tobacco plants. The detection limit of the test for purified, solubilized Bt2, was 0.1–1.0 ng ml^{-1}. Toxin levels in plant leaves are expressed as ng toxin per mg of total soluble protein. Insecticidal activity was determined using *Manduca sexta* larvae feeding on leaf material. Mortality after 3, 4, 6 days was determined and weight reduction in the surviving larvae measured after 6 days.

Our experiments illustrate the feasibility of engineering plants that defend themselves against lepidopteran insects which are sensitive to the *B. thuringiensis berliner* insect toxin. However some species, such as *Heliothis* and *Spodoptera* which belong to the *Noctuidae*, an important group of pest insects, are less sensitive to common strains of *B. thuringiensis*, including berliner 1715 (ref. 19). To protect plants fully against these insects, higher levels of expression will be required. This might be achieved using chimaeric *Bacillus* genes containing stronger plant-specific promoters. The 35S promoter of cauliflower mosaic virus[20], for example directs a 10–50-fold higher expression than the regular T-DNA promoters in plants. Alternatively, it may be possible to construct chimaeric toxin genes with higher specific activity against important target insects. Transfer of different chimaeric genes into a variety of crops may provide a new and environmentally safer method of controlling destructive insect pests.

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Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis berliner* 1715

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A plasmid-encoded crystal protein gene (*bt2*) has been cloned from *Bacillus thuringiensis berliner* 1715. In *Escherichia coli*, it directs the synthesis of the 130-kDa protein (Bt2) which is toxic to larvae of *Pieris brassicae* and *Manduca sexta*. Comparison of the deduced amino acid sequence of this Bt2 protein with the *B. thuringiensis kurstaki* HD1 Dipel, *B. thuringiensis kurstaki* HD73 and *B. thuringiensis sotto* crystal protein sequences suggests that homologous recombination between the different genes has occurred during evolution.

Treatment of the Bt2 protein with trypsin or chymotrypsin yields a 60-kDa protease-resistant and fully toxic polypeptide. The minimal portion of the Bt2 protein required for toxicity has been determined by analysing the polypeptides produced by deletion derivatives of the *bt2* gene. It coincides with the 60-kDa protease-resistant Bt2 fragment and it starts between amino acids 29 and 35 at the N-terminus and terminates between positions 599 and 607 at the C-terminus.

Bacillus thuringiensis is a gram-positive bacterium which produces endogenous crystals upon sporulation. The crystals are composed of proteins which are specifically toxic against certain insect larvae, mainly lepidopteran and dipteran species [1]. Upon ingestion by larvae, the crystals dissolve in the alkaline conditions of the insect midgut and release proteins of 130–160 kDa [2, 3]. Which are proteolytically processed by midgut proteases to yield smaller toxic fragments [4].

Most crystal protein genes have been localised on large plasmids [5, 6]. Some genes have recently been cloned and expressed in *Escherichia coli* [7–10]. However these cloned gene products have not been subjected to a detailed functional characterisation. On the other hand, all biochemical studies have been performed on proteins derived from the original crystals of *B. thuringiensis* [4, 11]. Generally such crystals are mixtures of distinct polypeptides which may exhibit different functional properties.

This paper describes the cloning of a crystal protein gene from *B. thuriengiensis* subspecies *berliner* 1715. The purified recombinant polypeptide was shown to exhibit a toxic activity to *Pieris brassicae* and *Manduca sexta* larvae comparable to the activity of the original crystal protein. The minimum polypeptide fragment still exhibiting complete toxic activity was mapped within the crystal protein.

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Abbreviations. B.t., Bacillus thuringiensis; SDS-PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; kb, 10^3 bases; ELISA, enzyme-linked immunosorbent assay; bp, base pairs; NaCl/P_i, phosphate-buffered saline; HPLC, high-performance liquid chromatography; LD_{50}, dose at which 50% lethality is observed.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *B. thuringiensis* strain *berliner* 1715 was kindly provided by Dr A. Klier (Inst. Pasteur, Paris) [8]. Growth and sporulation conditions were described in [12]. *E. coli* strains used were K514 [13] and K-12ΔH1Δtrp [14].

Plasmids used were pLK54, pLK85, pLK63 and pLK94 [15], pUC8 [16] pKM109/90 [17] and pEcoR251; the latter is a suicide vector expressing the *Eco*RI gene which can be inactivated by cloning DNA fragments in a unique *Bgl*II site, thus allowing a positive selection (Botterman et al., unpublished work).

Plasmids carrying a λP_L or λP_R promotor fragment were maintained in the cI-repressor-producing strain K514(λ). Temperature induction of the λP_L or P_R promoter was accomplished in strain K-12ΔH1Δtrp which carries a temperature-sensitive cI repressor, essentially as described by Zabeau and Stanley [14], except that induction was at 38 °C.

DNA MANIPULATIONS

Recombinant DNA techniques were as described by Maniatis et al. [18] and DNA sequencing was performed according to Maxam and Gilbert [19].

3'-End deletions in bt2

Fig. 1 shows pLBKM25, the intermediate plasmid used to construct the 3'-end deletion derivatives. This plasmid is composed of the following elements (the numbers between

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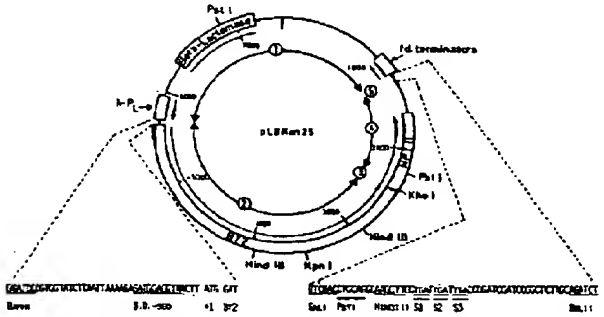

Fig. 1. *Intermediate plasmid pLBkm25, used to construct 3' end deletions in* bt2. The sequences at the 5' end of *bt2* and the region around the synthetic stop codons (S1-S2-S3) are shown. The numbers are explained in Materials and Methods

brackets refer to Fig. 1): a fragment of pLK54 carrying the β-lactamase gene, the origin of replication of pBR322, the lambda P_L promotor and two transcription terminators of phage fd in tandem (1); a fragment of pGL502 comprising the *bt2* gene from position −32 to 3240 in the DNA sequence (2); an *Xho*I linker (3); the *Hin*dIII-*Sal*I fragment of pKM109/90 containing the *neo* gene deleted at the 5' end up to the 5th codon (4); a fragment of pLK94 carrying three stop codons in the three reading frames to prevent read-through translation from an open reading frame fused to the *Sal*I site (5). pLBKM25 was treated with *Kpn*I, *Sal*I, *Pol*Ik and ligase to generate pLB16; with *Hin*dIII and ligase to produce pLB12 and with *Kpn*I, *Bal*31 exonuclease, *Sal*I, the 'Klenow' fragment of *E. coli* DNA polymerase I, and ligase to produce the other 3' deletion derivatives.

5'-End deletions in bt2

pRB10, derived from pGI502 and pLK63, contains the λP_R promotor, the upstream region and the ATG start codon of the *cro* gene followed by a *Bam*HI linker fused to the second codon of the full-length *bt2* gene. This gives rise to the sequence ATG GAT CCC GAT at the 5' end of the gene with GAT being the 2nd codon of *bt2*. pRB210 is derived from pRB10. The *bt2* gene has been deleted up to the 37th codon using the *Cla*I site at position 106. The sequence at the 5' end is: ATG GAT CGA TCC GAT where GAT is the 37th codon of *bt2*.

Fusions of bt2 *gene fragments to the 5' end of* lacZ

pBZ12 is derived from pLB879 and pLK85, whereby the *lacZ* gene has been fused to the 3' end of the deleted *bt2* gene, showing the sequence CCG GCA TCG ACC GAT CCC at the junction where T is position 1821 in *bt2* and C position 22 in *lacZ*. Similarly, pBZ13 is derived from pLB834 with the junction sequence being: TAT ATA GCA GAT CCG TCG ACC GAT CCC where G is position 1798 in *bt2* and C position 22 in *lacZ*.

BIOCHEMICAL METHODS

Preparation of crystal proteins

Crystals were isolated and purified from spore preparations as described by Mahillon and Delcour [12]. Crystal proteins were solubilized in 50 mM Na_2CO_3 pH 10, 5 mM dithiothreitol or in 50 mM $NaHCO_3$ pH 9.5, 200 mM thioglycolate. More than 90% of the crystal proteins were solubilized after a 2-h incubation at 37°C.

Purification of Bt2 protein

Cells (5 g) from a saturated culture of K514 (pGI502) pellet were resuspended in 50 ml 50 mM Tris/HCl pH 7.9, 50 mM EDTA, 15% sucrose, treated with lysozyme (100 μg/ml) and sonicated on ice (10 min, 400 W). The cell debris were washed twice with 200 ml NaCl/P_i containing 2% Triton X-100, incubated for 30 min in the same buffer at 0°C, centrifuged (10 min, 15000 × g) and washed twice with NaCl/P_i. The Bt2 protein, present in the pellet, was solubilized in 50 ml extraction buffer (0.1 M $NaHCO_3$, pH 9.5, 0.2 M thioglycolate) for 2 h at 37°C.

For structural analysis, the Bt2 was further purified. The pellet was dissolved in buffer A (50 mM Tris/HCl, pH 8.0, 1 M urea, 0.2% 2-mercaptoethanol) and loaded on a column (10 × 2.5 cm) of DEAE-cellulose (Whatman DE 52) pre-equilibrated in the same buffer, washed twice with 50 ml buffer A, and eluted with 100 ml of a 0−0.3 M KCl linear gradient in buffer A. Fractions containing the Bt2 protein were pooled and precipitated at pH 4. The precipitate was collected and redissolved in 3 ml buffer A pH 10. Then the pH was re-adjusted to 8; the sample applied to a Sephacryl S-300 column (100 × 2.5 cm) and eluted with a flow rate of 40 ml/h. Pure Bt2 protein was dialysed exhaustively against 0.1 M NH_4HCO_3 containing 0.2% 2-mercaptoethanol.

Generation of the 60-kDa tryptic fragment of Bt2

Purified Bt2 protein (1 mg/ml) was digested with bovine trypsin or chymotrypsin (Sigma) 1:70 for 45 min at 37°C.

Protein sequencing

Amino-terminal sequences were determined using a gas-phase sequenator (Applied Biosystems Inc. USA), operated essentially as described by Hewick et al. [20]. Stepwise liberated phenylthiohydantoin derivatives were identified by HPLC analysis as described by Hunkapiller and Hood [21]. The 60-kDa Bt2 fragment was separated on SDS-PAGE, electroblotted onto polybrene-coated glass-fibre sheets [22] and sequenced.

Immunodetection methods

Polyclonal antisera against crystal proteins and against Bt2 were raised in rabbits and mice. These antisera reacted strongly with Bt2 and with solubilized crystal proteins from *B.t. berliner* both in Ouchterlony assay and in ELISA.

Hybridomas producing monoclonal antibodies against *B.t.* crystal proteins will be described elsewhere (Vanderbruggen et al., unpublished results). Immunological screening of bacterial colonies [23] and immunoblotting on nitrocellulose [24] as performed using rabbit anti-(crystal serum) and a peroxidase-labelled goat anti-(rabbit Ig) (Sigma).

Quantification of soluble crystal proteins or derived polypeptides was carried out with the ELISA techniques [25]. Flexible polyvinyl microtiter plates, precoated with goat anti-(*B.t.* crystal protein) antibodies were incubated for 90 min at 4°C with dilutions of the antigen samples. After washing, a diluted anti-(*B.t.* crystal protein) antibody was added, usually a hybridoma culture supernatant (1 h at room temperature).

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Table 1. *Toxicity experiments*
The LD_{50} values for *P. brassicae* (3rd larval instar) and for *M. sexta* (1st larval instar) of different toxin preparations, were measured. n.t. = not tested

Toxin	LD_{50} for	
	P. brassicae	*M. sexta*
	ng/larva	ng/cm²
Bt crystal from *B.t. berliner*	15	n.t.
As above, solubilized	0.6	7.5
Bt2 protein	1.6	6
Bt2 protein/trypsin	1.5	5

Plates were washed and incubated with a dilution of alkaline-phosphatase-labeled anti-(mouse IgG) antibodies (Sigma A-5153). After washing, *p*-nitrophenyl phosphate (Sigma, 104-105) was added and the reaction monitored by measuring the *A* at 405 nm. The detection limit of the test for purified, solubilized crystal protein, was in the range 0.1 – 1 ng/ml. Screening of hybridoma supernatants was performed on microtiter plates which were coated directly by an overnight incubation with the crystal protein solution at 4°C (10 µg/ml).

Insect toxicity assays

Toxicity assays were performed on first instar larvae of the tobacco hornworm (*Manduca sexta*). 3 ml of artificial diet [26] without formaldehyde was dispersed in a 4-cm² vial; a 200-µl sample was applied and four newly hatched larvae were placed into each vial. 20 larvae were used per sample dilution. Growth and mortality were followed over a 7-day period.

Toxicity assays on larvae of the large white cabbage butterfly (*Pieris brassicae*) were done on discs (0.25 cm²), cut from fresh cabbage leaves (*Brassica oleracea* var. *gemnifera* D.C.) on which 5 µl of a sample dilution was applied. Third instar larvae were obtained from a synchronised culture of *P. brassicae*. When the first disc was consumed, a fresh disc without sample was given. Viability of 50 larvae per sample dilution were monitored every 24 h over a period of 5 days.

RESULTS

Cloning of a B. thuringiensis *gene encoding a 130-kDa crystal protein exhibiting insecticidal activity*

The SDS/polyacrylamide gel electrophoresis patterns of *B. thuringiensis berliner* 1715 crystal preparations show two major protein bands of about 140 kDa and 130 kDa (Fig. 2A, lane 10). The dissolved purified crystals from this strain are highly toxic towards *P. brassicae* and *M. sexta* (larvae (Table 1).

A library was constructed by cloning size-fractionated (10 – 15 kb) *Sau*3A-digested plasmid DNA from *B. thuringiensis berliner* 1715 into the suicide vector pEcoR251. Four colonies producing crystal proteins were identified among 1750 clones using a colony immunoblot assay with rabbit anti-(crystal protein) serum. Restriction enzyme analysis revealed that the four clones contained overlapping DNA fragments. From one of these clones, pGI612, a 7.5-kb *Bam*HI-*Pst*I fragment, comprising a region shared by the four plasmids, was subcloned into pUC8 to produce plasmid pGI502.

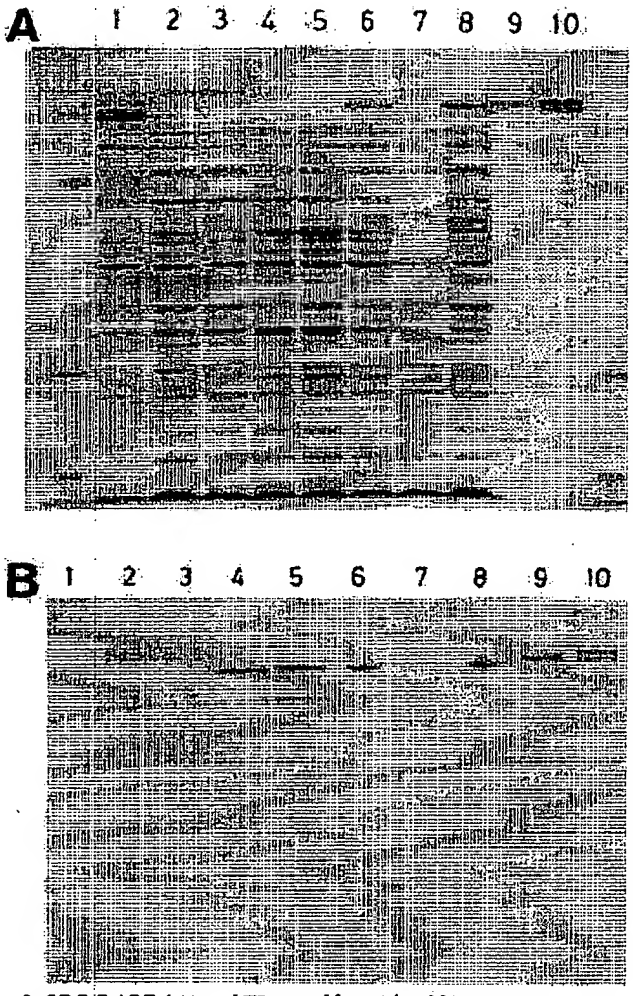

Fig. 2. *SDS-PAGE (A) and Western blot with rabbit antiserum against* B.t. berliner *crystal proteins (B) of clones containing the* bt2 *gene and its derivatives.* (A) A 10% polyacrylamide gel and Coomassie staining were used. Total cellular extracts of 2×10^8 cells were induced for 5 h. Lane 1, K-12ΔH1Δtrp (pHH10 control *lacZ*); lane 2, K-12ΔH1Δtrp (pBZ13); lane 3, K-12ΔH1Δtrp (pBZ12); lane 4, K-12ΔH1Δtrp (pRB210); lane 5, K-12ΔH1Δtrp (pRB10); lane 6, K-12ΔH1Δtrp (pLB10); lane 7, K-12ΔH1Δtrp (pLB10) grown at 28°C; lane 8, K514 (pGI502); lane 9, 2 µg of purified Bt2 protein from clone K514 (pGI502); lane 10, 3 µg *B.t. berliner* crystals. Standard molecular mass markers (BioRad) are on both side. (B) Lanes as in A but with different amounts: lanes 1 – 4, extracts from 2×10^8 cells; lane 5, 1×10^8 cells; lanes 6 – 8, 5×10^6 cells; lane 9, 0.2 µg purified Bt protein; lane 10, 0.3 µg *B.t. berliner* crystal protein

Total cell extract of *E. coli* K514 (pGI502) revealed an intense protein band with apparent molecular mass of 130 kDa on SDS-PAGE which was not present in K514 containing the pUC8 plasmid without insert. This protein, termed Bt2, comigrates with one of the major crystal proteins of *B.t. berliner* in SDS-PAGE (Fig. 2A, lane 8). It represented between 5% and 10% of the total protein content in K514 (pGI502). It was present as a precipitate in *E. coli* and could be solubilized under the conditions required to solubilize *B.t.* crystals. The relationship of purified Bt2 protein with *B.t.* crystal proteins was analysed: in Western blotting, Bt2 reacted

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strongly with a rabbit anti-(*B.t. berliner* crystal) serum (Fig. 2B, lane 9) and in an ELISA, 8 out of 16 monoclonal antibodies generated against *B.t. berliner* crystal proteins were reactive with the purified Bt2. Purified Bt2 showed toxicity levels comparable to those of solubilized crystals from *B.t. berliner* against *P. brassicae* and *M. sexta* larvae. (Table 1).

Nucleotide sequence of the toxin gene

Fig. 3 shows the restriction enzyme map of the 7.5-kb *Bam*HI-*Pst*I fragment from pGI502 containing the *bt2* gene. To localize the gene on this fragment, production of Bt2 protein by deletion mutants generated by the enzymes *Hpa*I, *Kpn*I and *Xba*I was monitored, using immunoassays. The *Hpa*I-deleted plasmid still encoded an intact 130-kDa protein indicating that the Bt2 toxin encoding sequence is localized on the 4343-bp *Hpa*I-*Pst*I fragment. The DNA sequence of this fragment (Fig. 3) shows one large open reading frame starting at an ATG codon at position 1 and ending at a TAA termination codon at position 3466. It codes for a protein of 1155 amino acids with a predicted molecular mass of 130533 Da, which agrees well with the molecular mass of Bt2 as determined in SDS-PAGE. Bt2 protein from K514 (pGI502) was additionally purified by DEAE-Sephadex ion-exchange chromatography and Sephacryl gel filtration. The amino acid sequence of the 20 N-terminal residues of this purified protein was determined by gas-phase sequencing [20]. This sequence, Xaa-Asp-Asn-Asn-Pro-Asn-Ile-Asn-Glu-Xaa-Ile-Pro-Tyr-Asn-Xaa-Leu-Xaa-Asn-Pro, is identical to the amino acid sequence deduced from the nucleotide sequence (Fig. 6) (Xaa indicates residues for which unambiguous identification was not possible).

A 60-kDa toxic polypeptide generated through proteolytic degradation of the 130-kDa Bt2 protein

The delta-endotoxins of *B.t.* are believed to be protoxins which are degraded by insect gut proteases into smaller active toxins [11]. We therefore investigated whether smaller toxic polypeptides could be generated from purified Bt2 by proteolytic cleavage with either trypsin or chymotrypsin. At defined time intervals, aliquots were analysed on SDS-PAGE. The 130-kDa Bt2 protein is rapidly degraded by trypsin or chymotrypsin and yields a major polypeptide of 60 kDa after a 10-min digestion at 37°C. This 60-kDa polypeptide is relatively resistant to further degradation by both enzymes (over a 2-h period), indicating that it constitutes a protease-resistant core within the Bt2 protein. The 60-kDa tryptic polypeptide was purified by gel filtration and its insect toxicity was determined. On a molar basis, it was equally toxic to *P. brassicae* larvae as intact 130-kDa Bt2 (Table 1). The N-terminal sequence of the 60-kDa tryptic core was determined by gas-phase sequencing as Ile-Glu-Thr-Gly-Tyr-Thr-Pro-Ile-Asp-Ile-Xaa-Leu allowing its unambiguous location in the original Bt2 sequence, starting from residue number 29 (see Fig. 5).

Delineation of the minimum gene fragment encoding an active toxin

Derivatives of the Bt2 gene that contain different deletions at the 5′ or the 3′ end were constructed and expressed in *E. coli*. We analysed the produced polypeptides to delineate the minimal Bt2 fragment required for insect toxicity.

The toxin gene was placed under transcriptional control of the λP_L promotor. The resulting plasmid pLB10 directed

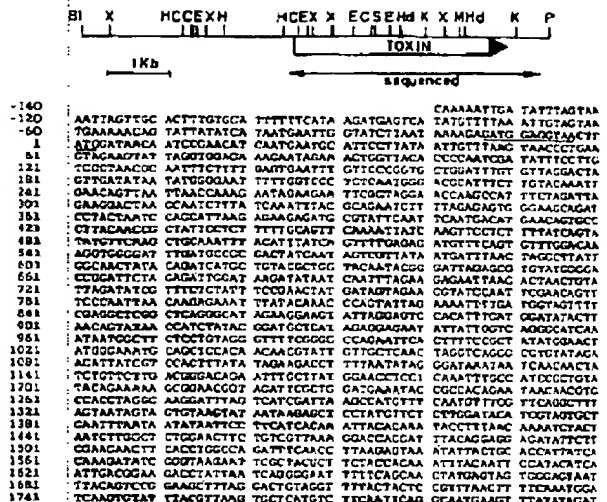

Fig. 3. *Restriction map of the* Bam*HI-*Pst*I fragment of* B.t. berliner *plasmid DNA in pGI502 (A) and the nucleotide sequence of the fragment (B)*. (A) The sequenced region is indicated. The arrow represents the toxin gene. X = *Xba*I; E = *Eco*RI; C = *Cla*I; Bl = *Bam*HI; H = *Hpa*I; Hd = *Hin*dIII; S = *Sac*I; K = *Kpn*I; M = *Mlu*I; P = *Pst*I. (B) The ATG initiation codon, the TAA stop codon and the Shine and Dalgarno sequence of *bt2* are underlined

high level expression of the Bt2 protein after induction (Fig. 2A, lanes 6 and 7; Fig. 2B, lanes 6 and 7). We subsequently constructed pLBKm25 (see Materials and Methods), a derivative of pLB10, which allowed the isolation of a series of 3′ deletions of the *bt2* gene. Fig. 4 shows the positions of the different deletion end points. One of these deletions (pLB16), comprises *bt2* gene sequences up to the *Kpn*I site at position 2170. *E. coli* K-12*Δ*H1*Δ*trp containing pLB16 produces a 80-kDa polypeptide reactive with anti-(*B.t. berliner* crystal) serum in Western blotting. When assayed on *P. brassicae* larvae, this protein exhibited toxicity levels similar to those of intact Bt2 protein (Table 2). In another deletion clone, pLB12, the *bt2* gene extends to the *Hin*dIII site at position 1692 and encodes a 60-kDa protein, still detectable as a faint band in Western blotting. However, extracts from this clone were completely nontoxic towards *P. brassicae* larvae (Table 2). These results suggest that the gene fragment

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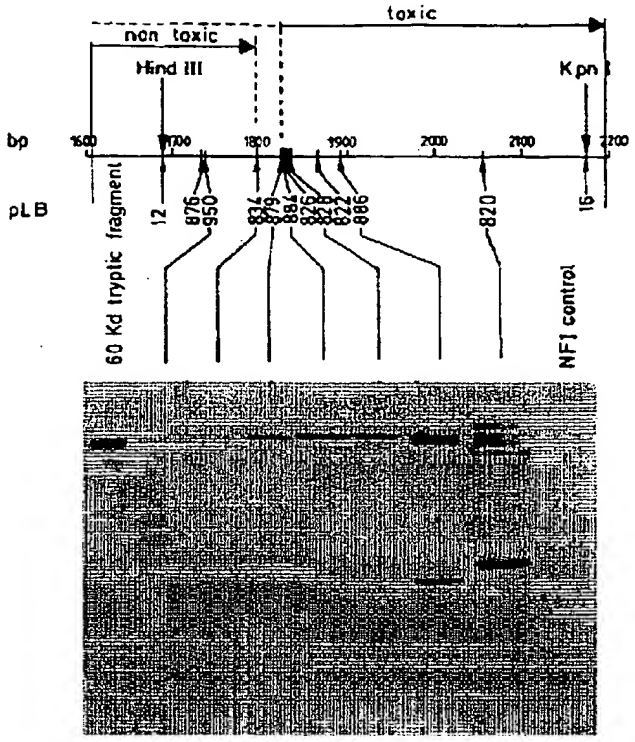
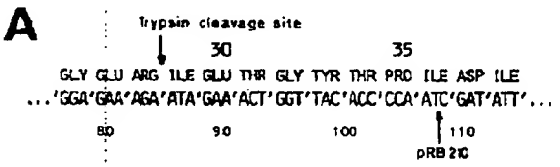


Fig. 4. *Localisation of the endpoints of the 3′ deletions in* bt2. Positions of the 3′ ends of deletion clones are indicated. Also shown is the result of a Western blotting with anti-(*B.t.* crystal) serum on extracts of some of these clones

Table 2. *Toxicity against 3rd instar larvae of* P. brassicae *of cell extracts of K-12ΔH1Δtrp strains containing different plasmids*
Toxicity was measured as percentage mortality after 4 days

Strain	Toxicity at dilution of		
	1	1/10	1/100
	%		
pLB16	100	100	6
pLB820	100	100	54
pLB822	100	100	80
pLB828	100	100	68
pLB826	100	100	96
pLB884	100	100	74
pLB879	98	50	8
pLB834	0	2	2
pLB950	6	0	2
pLB876	2	0	4
pLB12	0	0	0
pBZ12	100	74	22
pBZ13	4	0	0
pHH10	10	0	0
pRB10	100	100	100
pRB210	6	2	4



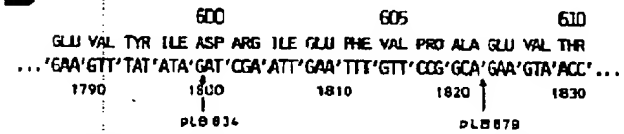


Fig. 5. *The sequence around the 5′ end (A) and the 3′ end (B) of the smallest toxin-encoding* bt2 *gene fragment.* The end points of *bt2* in the deletion clones and the N-terminus of the 60-kDa trypsin-cleavage product are indicated

encoding the active toxin is contained within the *Kpn*I deletion fragment but extends beyond the *Hin*dIII site. To determine the 3′ end point of the minimal fragment precisely, random deletion mutants encoding N-terminal fragments of decreasing size, starting from the *Kpn*I site, were constructed using exonuclease *Bal*31. Nine derivatives which have their deletion end points in the *Hin*dIII-*Kpn*I region were analysed. Extracts from K-12ΔH1Δtrp (pLB879) and from larger clones were fully toxic whereas extracts from K-12ΔH1Δtrp (pLB834) and from smaller clones were completely non-toxic to *P. brassicae* larvae (Table 2). The presence of Bt2-like polypeptides in the extracts was verified using Western blotting with anti-(*B.t. berliner* crystal) serum (Fig. 4) and revealed that less Bt2-like antigen was present in K-12ΔH1Δtrp (pLB834) than in K-12ΔH1Δtrp (pLB879).

However this quantitative difference could not account for the complete lack of toxicity of K-12ΔH1Δtrp (pLB834) over a 100-fold dilution range (Table 2). The deletion endpoints in pLB879 and pLB834 were determined by DNA sequencing (Fig. 5) and indicated that the critical end point of a DNA fragment encoding an active toxin maps between positions 1798 and 1821 on the *bt2* gene.

The lack of toxicity in pLB834 could result from a higher susceptibility to proteolytic degradation of the pLB834-endoded polypeptide. To investigate this possibility, we constructed inframe fusions of the *bt2* gene fragments contained in pLB834 and pLB879 to the 5′ end of *lacZ*, to produce pBZ12 and pBZ13 respectively. It has been shown that stable β-galactosidase fusion proteins can be easily produced in high quantities in *E. coli* [14, 27]. SDS-PAGE analysis showed that the *bt2-lacZ* fusion genes directed the production of high amounts of protein with a high molecular mass as expected for the fusion protein (Fig. 2A, lanes 2 and 3). Reactivity with anti-(*B.t. berliner* crystal) serum (Fig. 2B, lanes 2 and 3) and anti-(β-galactosidase) serum (not shown) in Western blotting confirmed the presence of both Bt2 and β-galactosidase determinants. The toxicity of pBZ12 was comparable to that of pLB879, whereas the equally stable fusion protein encoded by pBZ13 was completely nontoxic (Table 2).

To delineate the 5′ border of the gene fragment encoding an active toxin, we constructed pRB210 which contains a 5′ deletion in the *bt2* gene lacking the first 36 codons. A polypeptide encoded by pRB210 would start only eight amino acids beyond residue 29, the N-terminus of the fully active 60-kDa processed toxin. Clones containing pRB210 and pRB10, a control plasmid containing a full-length *bt2* gene, both produced proteins of the expected size that reacted with anti-Bt2 antiserum (Fig. 2A, lanes 4 and 5; Fig. 2B, lanes 4 and 5). Strain K-12ΔH1Δtrp (pRB10) was fully toxic whereas

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K-12ΔH1Δtrp (pRB210) was completely non-toxic to *P. brassicae* larvae (Table 2). Thus the N-terminus of the minimal toxic polypeptide is localized between amino acid positions 29 and 37 (Fig. 5).

Taken together, these data show that the minimal toxic fragment of the Bt2 protein is a 60-kDa polypeptide delineated by residues 29 and 37 at the N-terminus and amino acids 601 – 607 at the C-terminus (Fig. 5).

DISCUSSION

A plasmid-encoded crystal protein gene from *B.t. berliner* 1715 has been cloned and expressed in *E. coli*. The complete nucleotide sequence was determined and found to encode a polypeptide of 1155 amino acids. In *E. coli* this gene directs the synthesis of a 130-kDa protein, Bt2, which shows biochemical properties similar to the 130-kDa *B.t. berliner* 1715 crystal protein. The toxicity of purified Bt2 against *M. sexta* and *P. brassicae* larvae is similar to that of the original crystal proteins produced by *B.t. berliner* 1715.

Comparison of the complete amino acid sequence of Bt2 with other deduced amino acid sequences from cloned crystal proteins genes of *B.t.* strains *kurstaki* HD1 (Bt kur HDI) [28], *kurstaki* HD73 (Bt kur HD73) [10] and *sotto* (Bt sotto) [9] leads to the following observations (Figs 6 and 7).

a) The four toxins show a similar molecular mass (130 kDa) and exhibit extensive sequence homology.

b) Bt kur HD1 and Bt sotto are nearly identical over the total length of the published Bt sotto sequence (12 substitutions in 934 amino acids).

c) The four proteins are almost identical from residues 1 to 282 (I in Fig. 7).

d) From positions 283 to 458, Bt2 and Bt kur. HD73 are nearly identical (two substitutions in 174 amino acids, II_A in Fig. 7 but different from Bt kur. HD1 and Bt sotto at 65 positions (II_B in Fig. 7).

e) From residues 467 to 723, Bt2 is nearly identical to Bt kur. HD1 and Bt sotto (three substitutions in 266 amino acids, III_A in Fig. 7) but different from Bt kur. HD73 (III_B in Fig. 7), particularly between positions 467 and 611 (94 substitutions in 144 residues).

f) From positions 724 to 1155, Bt kur. HDI is almost identical to Bt kur. HD73 (three substitutions 431 amino acids, IV_B in Fig. 7) but different from Bt2 (IV_A at position 793 with an apparent deletion of 25 residues in Bt2 and between amino acids 1054 and 1117 where 18 out of 63 residues differ). Interestingly, the corresponding 75-bp sequence which is deleted in *bt2* is flanked by an 8-bp direct repeat of the sequence (AAAGTGTG) in the other three genes. Such a direct repeat might allow excision of the fragment by homologous recombination, leaving one copy of the repeat as is found in the *bt2* sequence, except for a 1-bp substitution (position 2379). Alternatively, such direct repeats could arise from an insertion event.

The general picture emerging from this sequence comparison is represented in Fig. 7 and shows that stretches of nearly identical sequences can be identified, which are recombined in different ways in the respective 130-kDa polypeptides. Thus, the crystal protein genes in the various *B.t.* strains may have evolved through homologous recombination events giving rise to toxins with distinct structural and possibly also functional properties. Homologous recombination between different crystal genes is likely to occur since they are located on transmissible plasmids [29]. The observation that IS elements flank the crystal genes in *B.t. berliner* 1715 [12] and *B.t. kurstaki* HD1 [30] also suggests a high mobility of these genes.

Despite considerable differences between the coding sequences, the 5′ regions upstream of the initiation codon are completely identical in the four genes over at least 140 bp. This suggests a similar regulation of the expression of all four genes.

Hydrophobicity analysis of the deduced amino acid sequence of Bt2 (Fig. 7) reveals that the whole N-terminal half of the Bt2 protein is more hydrophobic than the C-terminal half. In addition, two highly hydrophobic regions are present near the N-terminus of the protein (amino acid positions 29 – 80 and 137 – 172). Whether they play a critical role in the toxin/cell membrane interaction, as was found for other protein toxins [31], remains to be determined.

Large-molecular-mass *B. thuringiensis* crystal proteins have been shown to be 'protoxins' which are processed by insect gut proteases. The *in vitro* digestion of these crystals, using either insect midgut juice or well defined proteolytic enzymes, has been described and a variety of toxic polypeptides ranging in molecular mass from 160 kDa [2] to as small as 1 kDa have been reported [32, 33]. Other data indicate that polypeptides in the 55 – 70-kDa range can be generated by proteolytic degradation from the original crystal proteins using different enzymes [4, 34], or by spontaneous degradation of solubilized crystal proteins [35]. We have purified a polypeptide of 60 kDa, obtained by tryptic digestion of the Bt2 protein, showing the same toxicity as the intact Bt2 protein. The N-terminus matches the Bt2 sequence starting from amino acid 29. Nagamatsu et al. [34] also isolated a trypsin-resistant toxic core peptide of 58 kDa from *B.t. dendrolimus*. The N-terminal amino acid sequence was identical to the one determined for the 60-kDa Bt2 fragment.

To determine the minimal gene fragment still encoding an active polypeptide toxin we have used deletions of the *bt2* gene. The minimal fragment was found to be an approximately 60-kDa polypeptide, delineated at its N-terminus between amino acid positions 29 and 37 and at its C-terminus between positions 599 and 607. Interestingly, this fragment largely overlaps with the trypsin-resistant 60-kDa polypeptide. Indeed two putative trypsin-cleavage sites are present which could give rise to an approximately 60-kDa polypeptide, starting at position 29, namely Arg-601 and Arg-619. Our data are in agreement with those of Schnepf et al. [28] who also localized the active toxic fragment from a cloned *B.t. kurstaki* crystal protein, in the N-terminal half of the protein.

Based on the data obtained with the different deletions, we conclude that removal of a few amino acids from either the N-terminus or the C-terminus of this 60-kDa polypeptide results in a complete loss of toxicity. The absence of toxicity observed in C-terminal deletions up to position 599 is also associated with a relative protease sensitivity of these truncated Bt2 proteins. This suggests that toxicity requires a specific three-dimensional conformation which is disturbed by deleting only a few terminal residues.

The generation of very small toxic peptides by insect gut proteases has been reported [20, 21]. However, our present data show that it is very unlikely that active toxic fragments smaller than the 60-kDa fragment can arise from the Bt2 protein. Experiments are now in progress to identify the functional domains of the toxin using monoclonal antibodies, specific for well defined regions on the Bt2 molecule.

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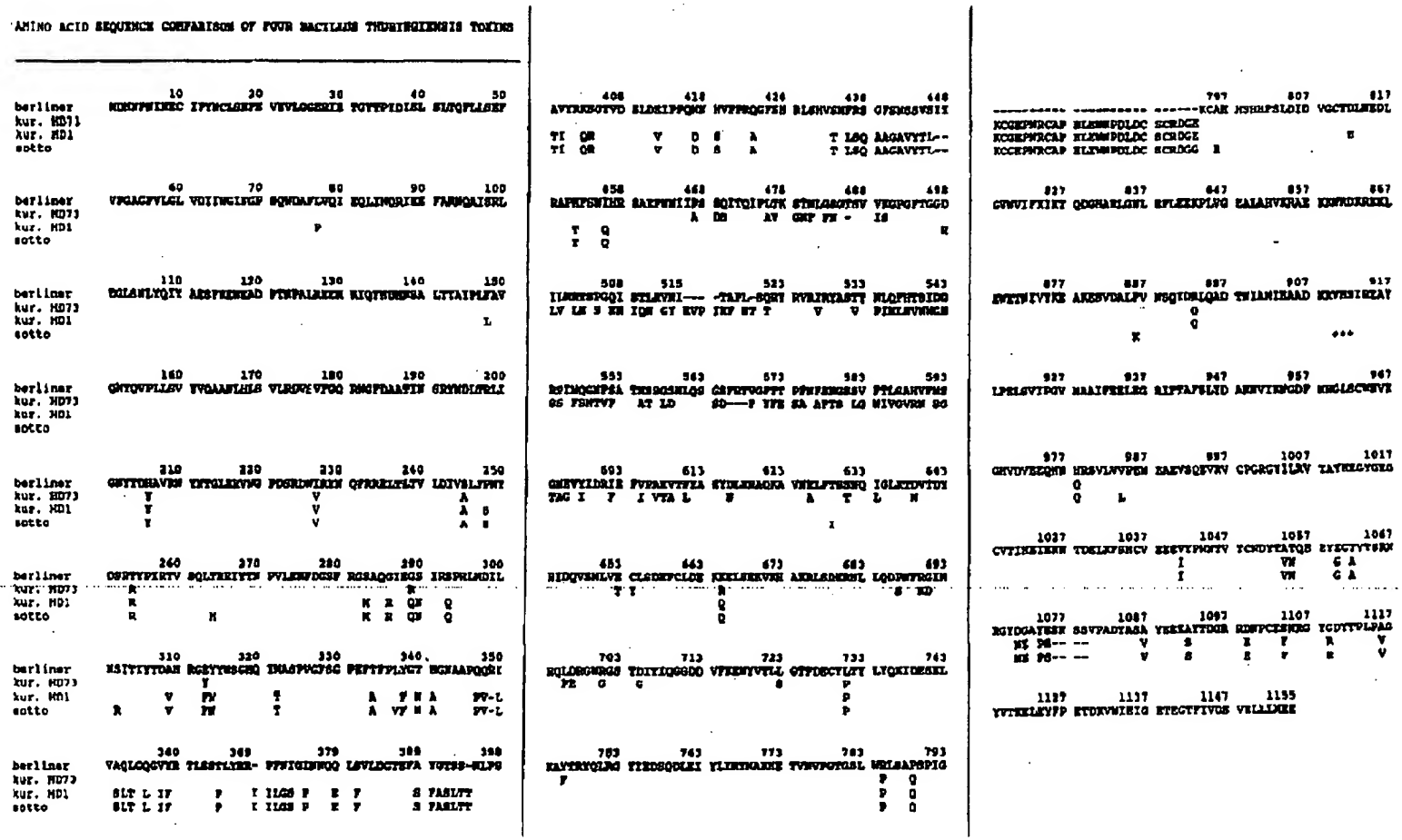

Fig. 6. *Amino acid sequence comparison of Bt2 and three other crystal proteins.* Sequences were aligned to the Bt2 sequence by adding gaps (indicated by –) where necessary. Amino acids in the other proteins are shown only when differing from the corresponding amino acids in Bt2. berliner = Bt2; kur. HD73 = *B.t. kurstaki* HD73 [10]; kur. HD1 = *B.t. kurstaki* HD1 [15]; sotto = *B.t. sotto* [9]

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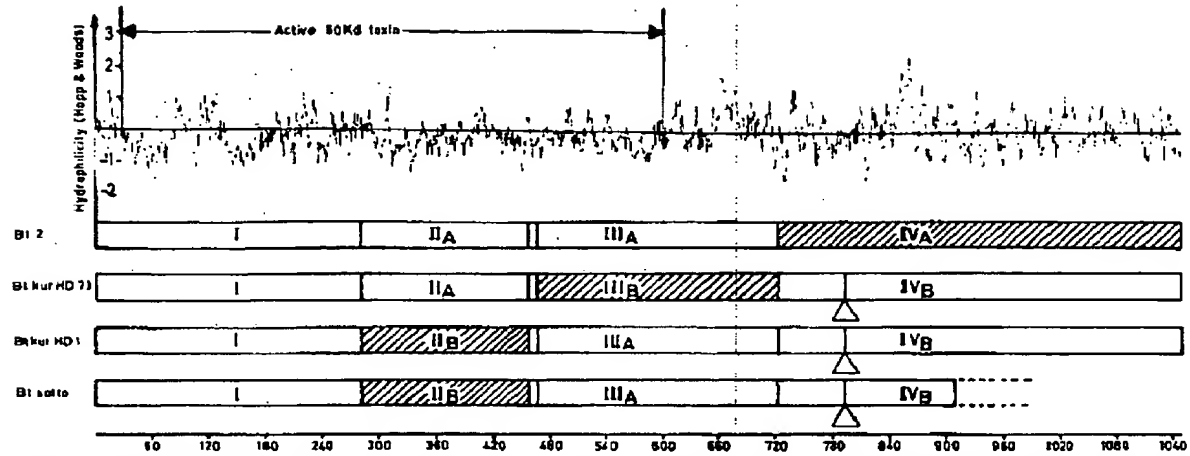

Fig. 7. *Diagram of the amino acid sequence comparison between the four crystal proteins and the hydrophilicity plot of Bt2 according to Hopp and Woods [36].* The location of the smallest active toxin in the amino acid sequence is shown. The roman numbers I—IV refer to the sequence 'blocks' mentioned in the text. The triangle indicates the sequence of 25 amino acids not present in Bt2. The hatched areas represent the amino acid stretches showing features unique for the protein considered

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Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn*5*

(Plasmids; kanamycin resistance gene; selection marker; homologies with Tn*903*)

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SUMMARY

The nucleotide sequence of 1200 bp from the unique region of transposon Tn*5* containing the neomycin phosphotransferase gene (*neo*) was determined, and the location of the *neo* gene was identified by deletion mutants in a translational reading frame of 792 bp. The derived gene product, an aminoglycoside 3′-phosphotransferase (APH) II, consists of 264 amino acid residues and has a calculated M_r of 29053. Its amino acid sequence shows sequence homologies to the APH type I enzyme coded for by transposon Tn*903* (Oka et al., 1981).

INTRODUCTION

Transposon Tn*5* carries a gene that confers resistance to the aminoglycosides kanamycin and neomycin. This gene (*neo*) codes for an aminoglycoside 3′-phosphotransferase II (Berg et al., 1975) and lies adjacent to the left inverted repeat of Tn*5* that has already been sequenced (Auerswald et al., 1981). The *neo* gene has been used as a selection marker for vectors in prokaryotes such as *Escherichia coli* (Rao and Rodgers, 1979; Herrmann et al., 1980) and *Bacillus subtilis* (Sprengel, 1982). Recently, this gene was also found to be a useful dominant selection marker for transformation of eukaryotic cells (Jimenez and Davies, 1980; Colbère-Garapin et al., 1981; Southern and Berg, 1982). However, only the approximate position of the *neo* gene in Tn*5* was known requiring use of relatively large DNA pieces to clone the antibiotic resistance marker. We have now determined the nucleotide sequence and the exact location of the gene so that it can be used for vector constructions defined at the nucleotide level.

MATERIALS AND METHODS

(a) Bacterial strains

Phage *fd*::Tn*5*-30 (Auerswald et al., 1981) was used as source of Tn*5* for all plasmid construc-

Abbreviations: APH, aminoglycoside 3′-phosphotransferase II; bp, base pairs; *neo*, neomycin resistance gene; pos, position in the sequence; SDS, sodium dodecyl sulfate.

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tions. Maxicell strain was CSR603 (Sancar et al., 1979).

(b) Enzymes

Restriction endonucleases (*Hae*III, *Hin*fI, *Eco*RI, *Hin*dIII) were prepared essentially as described by Roberts et al. (1979) or purchased from New England Biolabs (*Ava*I, *Ava*II, *Bgl*II, *Pvu*II, and *Sal*I). Cleavage buffer for all these restriction endonucleases was 20 mM Tris · HCl pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol and 5% glycerol. The DNA concentration in the assay was between 100 and 200 µg/ml. T4 polynucleotide kinase was purified by a modification of the procedure of Richardson (1965); S1 and BAL31 exonucleases were purchased from New England Biolabs and Bethesda Research Laboratories, respectively; calf intestinal phosphatase and T4 DNA ligase were from Boehringer Mannheim. Ligation buffer was 50 mM Tris · HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM ATP, 1 mM dithiothreitol, and 1 mM EDTA.

(c) Protein size marker

A ^{14}C-methylated mixture of lysozyme (M_r = 14 000), carbonic anhydrase (M_r = 30 000), ovalbumin (M_r = 46000), bovine serum albumin (M_r = 69000), phosphorylase b (M_r = 92000), and myosin (M_r = 200 000) was purchased from Amersham-Buchler, Braunschweig.

(d) Sequencing procedures

DNA sequencing was performed essentially as described by Maxam and Gilbert (1980). Sequencing gels were dried in order to enhance band sharpness and to shorten exposure times (Garoff and Ansorge, 1981). Computer programs of Osterburg et al. (1982) were used for storage and processing of sequences.

(e) Plasmids

(1) Plasmid pKm1 contains the complete *neo* gene on a *Hin*dIII-*Bam*HI fragment of Tn*5* cloned into pBR322 via the corresponding restriction endonuclease sites. An analogous construction was reported by Rao and Rodgers (1979).

(2) Plasmid pKm2 (see Fig. 1) is a derivative of pKm1 and expresses the *neo* gene under control of the *lac*UV5 promoter. The vector in this construction is a pBR322 derivative, pEX205, in which the short *Eco*RI-*Hin*dIII fragment of pBR322 is replaced by a 214-bp *Eco*RI-*Hin*dIII fragment carrying the *lac*UV5 promoter/operator control region (Reiss, 1982). This element consists of a 207-bp *Eco*RI fragment (Backman et al., 1976) with an *Eco*RI-*Hin*dIII adaptor element added at its 3'-end to yield the sequence: 5' GAATTCCAAGCTT 3'. Between the *Hin*dIII site and the *Sal*I site of this vector the *neo* gene was fused as follows: 1 pmol pEX205 DNA was cleaved with *Hin*dIII, precipitated with ethanol, the precipitate dried, redissolved in 20 µl of 30 mM sodium acetate pH 4.6, 280 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol and incubated with 0.1 unit of S1 nuclease for 10 min at 20°C to obtain an adenine nucleotide at the 3'-end. The nuclease digestion was terminated by the addition of 30 µl 10 mM EDTA, the sample was extracted with phenol, desalted by chromatography on Sephadex G-150 in 10 mM ammonium bicarbonate, lyophilised, and digested with *Sal*I. 1 pmol DNA of plasmid pKm1 was cleaved with *Bgl*II, precipitated with ethanol, redissolved in 20 µl 20 mM sodium phosphate buffer pH 7.2, 7 mM $MgCl_2$, 100 mM KCl, 100 µM each of all four deoxyribonucleoside triphosphates, and the cleavage site filled in with 2 units of *E. coli* DNA polymerase I for 10 min at 20°C. The resulting *neo* fragment was ligated to the linearised vector via the created blunt ends and *Sal*I sites in 30 µl of ligation buffer for 16 h at 15°C with 0.6 units T4 DNA ligase. Joining of the filled in *Bgl*II site of the *neo* gene to the S1-treated *Hin*dIII site of the pEX205 vector restored the *Bgl*II site in front of the *neo* gene in the sequence: 5' GATTCCAGATCT 3' (see Fig. 5).

(3) Plasmids pKm21 and pKm22. For the construction of these two kanamycin-sensitive plasmids 1 pmol of plasmid pKm2 DNA was opened with *Bgl*II, dissolved in 20 µl of 100 mM potassium phosphate pH 7.0, 100 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol and incubated at 20°C with 10 units of *E. coli* exonuclease III. After 30 s the sample was precipitated with ethanol and dried. The DNA was incubated with S1 nuclease as

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described above, extracted with phenol and desalted. An aliquot of the sample (0.1 pmol) was incubated with 20 pmol *Hin*dIII linker fragment (Collaborative Research, Waltham; MA) and 3 units of T4 DNA ligase in 10 µl of ligation buffer for 16 h at 15°C, then quickly heated at 65°C, diluted with 20 µl cleavage buffer and digested with 10 units *Hin*dIII for 1 h at 37°C. Linker fragments and salts were removed by chromatography on Sephadex G-150 and the DNA was circularised by ligation. Transformed C600 cells were screened for sensitivity to kanamycin and for the presence of the *lac* promoter on X-gal plates (Backman et al., 1976).

(4) Plasmid pKm24 is a derivative of plasmid pKm2 in which a 940-bp *Ava*I fragment downstream of the putative C-terminus of the *neo* gene has been deleted (see Fig. 1). For its construction 1 pmol of plasmid pKm2 DNA was digested with *Ava*I and the resulting two fragments were separated by agarose gel electrophoresis. The bigger 4.2-kb fragment was eluted from the gel and circularised by ligation. The ligation reaction worked sufficiently well although the two *Ava*I sites differed by one nucleotide (CCCGGG and CTCGGG, respectively).

(5) Plasmid pKm241 to plasmid pKm245 are deletion derivatives of plasmid pKm24 and were constructed as follows: 1 pmol of plasmid pKm24 DNA linearized by cleavage with *Ava*I was incubated at 25°C with 1 unit BAL31 exonuclease in 100 µl of 20 mM Tris·HCl pH 8.1, 600 mM NaCl, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 1 mM EDTA. After 2, 5, 10, and 20 min aliquots of 25 µl were withdrawn, phenol-extracted and desalted on Sephadex G-150. Half of each sample (0.12 pmol) was joined with *Hin*dIII linker fragments as described above for the plasmids pKm21 and pKm22 and recircularised. Ampicillin-resistant transformants were analysed on kanamycin plates. The 5- and 10-min aliquots of the BAL31 digestion resulted in about equal numbers of kanamycin-resistant and kanamycin-sensitive colonies. The plasmids were analysed for the ends of the Tn*5* DNA by cleavage with *Eco*RI and *Hin*dIII. For two kanamycin sensitive plasmids (pKm241 and pKm242) and three kanamycin-resistant plasmids (pKm243, pKm244, and pKm245) these ends were exactly determined by DNA sequence analysis.

RESULTS

To sequence the *neo* gene and to determine its limits, deletion mutants were constructed that contain the gene on a conveniently small piece of DNA. Preliminary sequence data had predicted that the N-terminus of the *neo* structural gene was located very close to the end of the left inverted repeat of Tn*5* next to a unique *Bgl*II site (Auerswald et al., 1981). Therefore, a (presumably) promoterless *Bgl*II-*Sal*I DNA fragment (1050 bp) containing the structural gene (see Fig. 1) was cloned under control of the *lac*UV5 promoter in a derivative of pBR322 (for details see MATERIALS AND METHODS) to give plasmid pKm2. This plasmid conferred a level of antibiotic resistance on *E. coli* host cells similar to that induced by the plasmids containing the intact transposon Tn*5*. In contrast, no antibiotic resistance was induced in a derivative of pKm2 lacking the *lac* promoter element. These results support the predicted position of the structural gene for APH in the *Bgl*II-*Sal*I subfragment and the absence of a promoter-element.

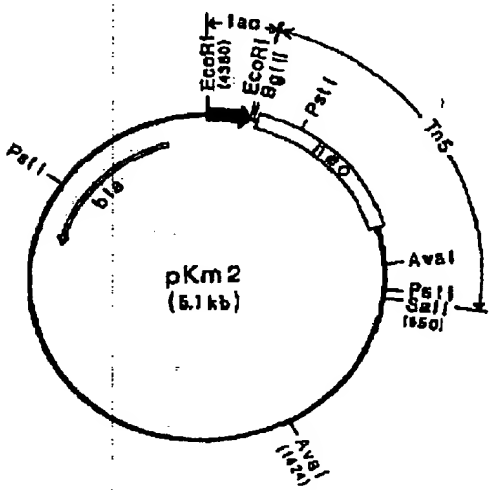

Fig. 1. Plasmid pKm2. The diagram shows size and position of the cloned Tn*5* DNA fragment, of the APH coding region (*neo*), and the *lac* promoter (heavy arrow) of the vector-encoded β-lactamase (*bla*), and of some important restriction endonuclease cleavage sites. Nucleotide positions of those sites in plasmid pBR322 are specified in parentheses.

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(a) The DNA sequence

The nucleotide sequence of the Tn*5* *neo* gene region was derived by the chemical method using cleavage sites of infrequently cleaving restriction endonucleases like *Aoa*I, *Aoa*II, *Bal*I, *Bgl*II, *Pou*II, and *Sal*I. An outline of the sub-sequences obtained is presented in the lower part of Fig. 2. In addition to double-stranded DNA fragments from plasmid pKm2, single-stranded Tn*5* DNA obtained from the single-stranded DNA phage fd::Tn*5* (Herrmann et al., 1978) was used for sequencing. In this DNA the inverted repeats of Tn*5* form a DNA double strand whereas the unique regions of the transposon and of all fd-specific sequences appear as loops at both ends of this "stem" structure. *Bgl*II endonuclease cleaves the base-paired part of this structure very close (19 bp) to the unique single-stranded region of Tn*5*, and thus very close to the *neo* gene. We have used this for a simplified sequencing strategy. After cleavage of fd Tn*5* DNA with *Bgl*II and 5′-end labeling with polynucleotide kinase the smaller of the two generated fragments could be used directly for DNA sequence analysis of the 5′-coding region of the *neo* gene without secondary cleavage by another restriction enzyme.

DNA fragments with only one labeled end were also obtained directly from the single-stranded loop region after cleavage with *Hae*III, whereas sequences in the stem region (up to pos. 1530) and beyond the *Sal*I site (pos. 2684) were determined using fd Tn*5* DNA of the double-stranded replicative form. Altogether a nucleotide sequence of 1300 bp was obtained which was determined over all the regions in both strands (Fig. 2). The complete nucleotide sequence is shown in Fig. 3. The first 300 bp (pos. 1400–1700) overlap with the DNA sequence of the inverted repeats of Tn*5* published earlier (Auerswald et al., 1981). There were no discrepancies except for a CC sequence at pos. 1682 which had to be corrected to CCC.

The DNA segment sequenced is relatively rich in CG (60%) and therefore contains many CG-rich cleavage sites for restriction endonucleases such as *Hpa*II, *Hha*I, and *Hae*III. Also a high preference for C or G as a third base of the codons (63%) was found. There are many stretches of self-complementary nucleotide sequences in the region of the *neo* gene. Examples of six and more base pairings can be recognized between pos. 1479 and 1494, 1752–1767, 1846–1861, 1908–1922, 2089–2107, 2199–2216, 2334–2354. This could contribute to a stable secondary structure of *neo* mRNA. Com-

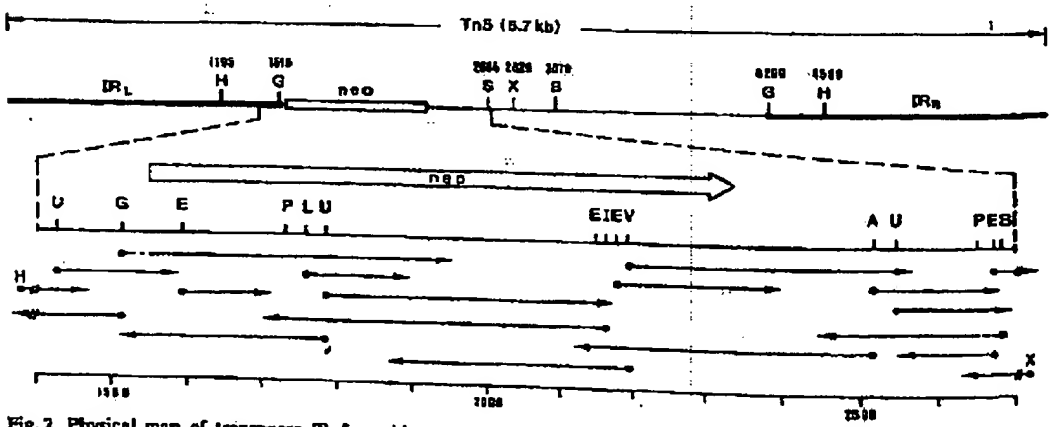

Fig. 2. Physical map of transposon Tn*5*, position of the *neo* gene and the sequencing strategy applied. The upper line shows transposon Tn*5* with the left and right inverted repeats (IR_L and IR_R), the *neo* gene (*neo*), and some restriction endonuclease cleavage sites. Positions of restriction sites above pos. 2700 are only approximate as the DNA sequence has not been determined yet sequence presented here (A = *Aoa*I; B = *Bam*HI; B = *Hae*III; G = *Bgl*II; H = *Hin*dIII; I = *Hin*fI; L = *Bal*I; P = *Pst*I; U = *Pou*II; V = *Aoa*II; S = *Sal*I; X = *Xba*I). Arrows in the lower part of the figure indicate the directions and extents of the sequencing runs. Numbering in the map reflects the published DNA sequence of the inverted repeats of Tn*5* (Auerswald et al., 1981).

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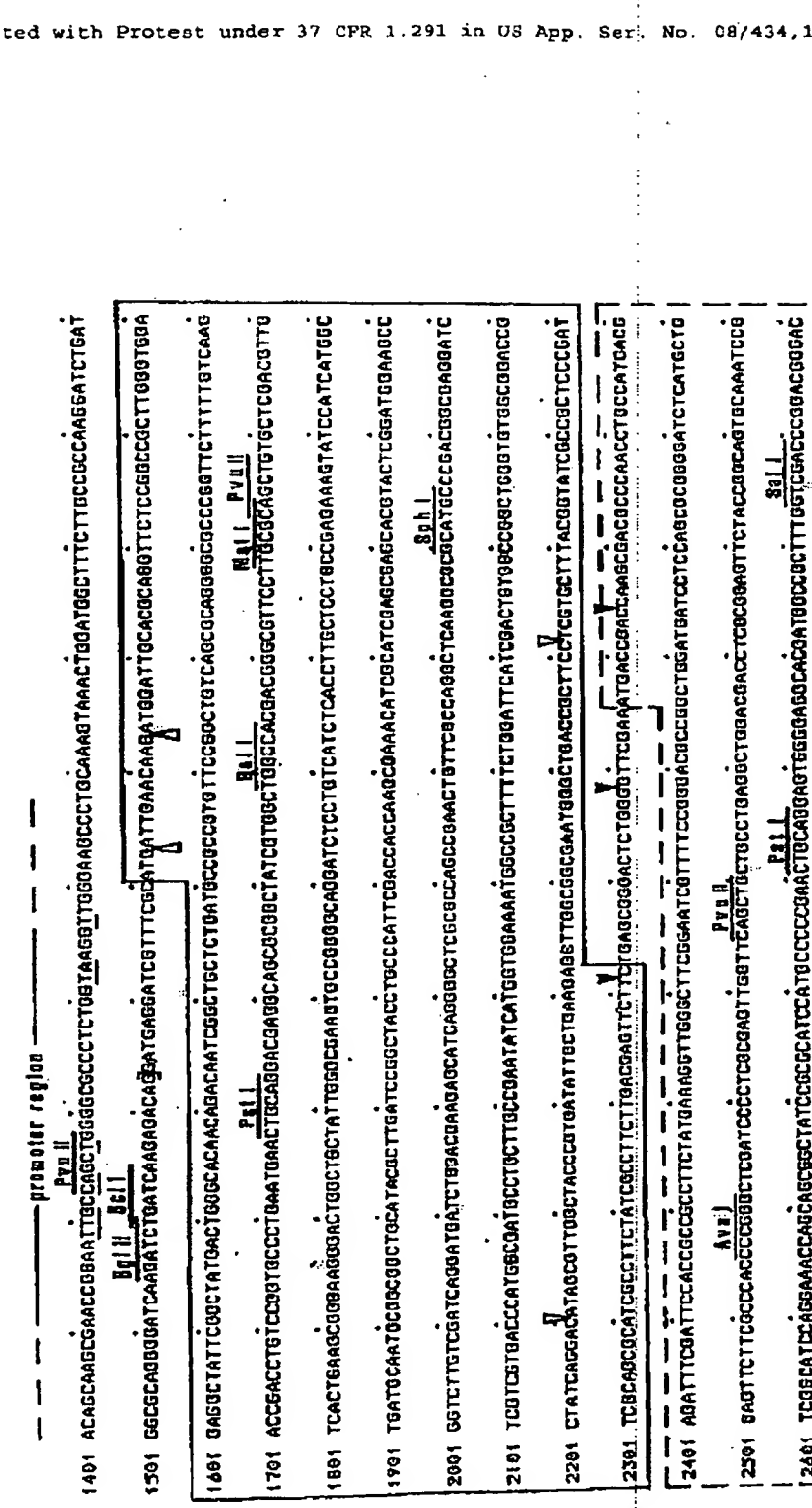

Fig. 3. Nucleotide sequence of Tn5 DNA from position 1401 to position 2700. The APH coding region is boxed by solid lines. A continuous reading frame coding for a postulated second protein is boxed by a broken line. Indicated in the sequence are some essential restriction sites, the end of the left inverted repeat (closing bracket,]), and the putative promoter region. Similarities between the model sequence (Reinberg and Court, 1979) and the new promoter proposed by Rothstein and Reznikoff (1981) are underlined. Endpoints of the Tn5 DNA in the kanamycin-sensitive 5'-deletion mutants pKm21 and pKm22 (see also Fig. 5) and 3'-deletion mutants pKm241 and pKm242 are indicated by upward and downward open arrowheads (Δ and ∇), respectively and endpoints of the kanamycin-resistant 3'-deletion mutants pKm243, pKm244, and pKm245 by filled arrowheads ($\blacktriangledown$).

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found by comparing calculated and experimentally determined amino acid contents for amino acids typical for protein ends such as phenylalanine and tyrosine. A smaller protein species ($M_r = 25000$) was sometimes detected in polyacrylamide gels in maxicell experiments upon prolonged incubation. However, the active enzyme, as identified by in situ phosphorylation of kanamycin on a native acrylamide gel (B. Reiss, R. Sprengel, and H. Will, in preparation), corresponded to the longer, M_r 27500 protein band on SDS gels.

(c) Deletion at the 5'-terminus of the *neo* gene

To determine the structural requirements at the 5'-terminus of the gene, plasmid pKm2 was cleaved with *Bgl*II and subjected to limited exonucleolytic degradation. To facilitate subsequent analysis, *Hin*dIII decanucleotide linkers were added before recircularization by ligation. Nucleotide sequences from two such constructions, pKm22 and pKm21, in which 36 and 46 bp of the Tn*5* DNA have been deleted in front of the *neo* gene are shown in Fig. 5. These deletions remove one or five codons from the 5'-end of the putative *neo* gene in pKm22 or pKm21, respectively. Despite the presence of a functional *lac* promoter both pKm22 and pKm21 lost the ability of their parental plasmid pKm2 to confer kanamycin resistance to host cells. This shows that expression of the *neo* gene depends on the presence of a relatively small DNA sequence (approx. 40 bp) deleted at the 5'-end including the ATG codon at pos. 1551.

To prove that the deletions had removed only the translation initiation signal derivatives of plasmid pKm22 were constructed in which the body of the gene was fused to the beginning of the *lacZ* gene contained in the *lac* element of pKm22 (see Fig. 2). In these fusion mutants kanamycin resistance was restored (Reiss, 1982). Both the loss of kanamycin resistance after deletion of the first ATG codon, and the restoration of gene activity after fusion with another start signal demonstrate that the coding region of the *neo* gene starts at pos. 1551. Additional independant evidence for this position comes from determination of the N-terminal amino acid sequence of the APH enzyme (J. Davies, personal communication). The sequence obtained, Met-Ile/Gly-Glx-X-Leu-X-Ser, agrees with the one predicted from the nucleotide sequence starting at pos. 1551. We therefore conclude that this is the start of the *neo* gene from transposon Tn*5*.

(d) Deletions at the 3'-end of the gene

A number of deletion derivatives were constructed to determine which part of DNA is essential for APH function around the 3'-terminus of the gene. Initially an *Ava*I fragment downstream of the presumptive C-terminus and extending from pos. 2516 in Tn*5* to pos. 1424 in pBR322 (see Fig. 1) was removed from plasmid pKm2. The resulting plasmid, pKm24, showed normal levels of kanamycin resistance and was used to construct further deletion mutants. For this purpose pKm24 was opened at the *Ava*I site, treated with BAL31 exonuclease, and the DNA recircularized after addition of *Hin*dIII linkers. Kanamycin-resistant and kanamycin-sensitive transformants were isolated and the respective plasmids analysed for the ends of the Tn*5* DNA sequence. As shown in Fig. 3 deletion endpoints determined in five plasmids were found to scatter around the predicted C-terminus of the gene at pos. 2344: Plasmids from kanamycin-resistant colonies (pKm241, pKm242, and pKm243) had endpoints at pos. 2374, 2358, and 2341, kanamycin-sensitive colonies (pKm244 and pKm245) at pos. 2271 and 2210, respectively. This shows that the DNA coding for the last 22 C-terminal amino acid residues of the APH enzyme cannot be removed without loss of kanamycin resistance. However, the Tn*5* sequence following the *neo* structural gene appears not to be essential for efficient transcription and translation of the gene; in pKm243 removal of all of the nontranslated sequence including the TGA stop codon did not lower the level of enzyme activity in extracts of transformed *E. coli* cells (Reiss, 1982). No transcription termination signal has been detected in this part of the nucleotide sequence.

DISCUSSION

This report presents the nucleotide sequence of about 1200 bp from the unique region of transpo-

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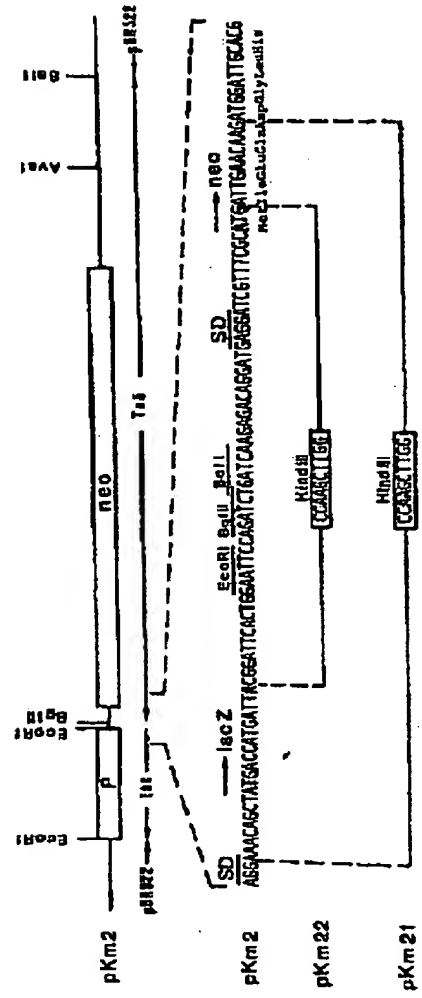

Fig. 5. DNA sequence at the junction site of *lacUV5* promoter and *neo* gene coding region of plasmid pKm2 and its deletion derivatives pKm21 and pKm22. The upper line illustrates the positions of *lac* promoter and *neo* gene. The translational start sites of *neo* and *lacZ* are indicated by arrows and the Shine–Dalgarno sequences by SD. The first eight amino acid residues of APH are shown. Deletions and the replacing linker fragments introduced in plasmids pKm21 and pKm22 are indicated in the lower part of the figure by solid lines and boxes, respectively.

Fig. 6. Comparison of APH amino acid sequences of Tn*5* and Tn*903* (Oka et al., 1981). The sequences are aligned for maximal homology. Asterisks indicate identical amino acid residues, circles indicate amino acid changes that remain in the same R-group and whose mutational distance is one base change.

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son Tn*5* which is one of the most actively studied transposable elements in prokaryotes. Thus, together with the sequence of the two inverted repeats (Auerswald et al., 1981) about 75% of the Tn*5* sequence has been established. As indicated in Fig. 3 we have identified the exact limits of the *neo* gene. In addition, there are several other open reading frames of unknown function. One, starting from position 2366 extends beyond the limits of our sequence and codes for more than 110 amino acids. In analogy with other transposons (e.g. Tn*3*; Chou et al., 1979) one would expect this putative gene to code for a regulatory protein for Tn*5* transposition. Two other open reading frames overlap with the coding region of the *neo* gene and code for hypothetical proteins of 52 amino acid residues (pos. 1564 to pos. 1719), and 126 amino acid residues (pos. 1723 to pos. 2106), respectively. We do not know whether these proteins are produced in vivo. However, no proteins of corresponding lengths were synthesized in detectable amounts from the cloned *neo* DNA in the maxicell system (see Fig. 4).

It has been speculated that transposon Tn*5* evolved from a fusion of a resistance gene with two copies of an independent IS element (Auerswald et al., 1981; Berg et al., 1981). If this was the case one would expect differences in CG content or in codon usage between the DNAs of the *neo* gene in the unique region and the transposase gene in the left inverted repeat of Tn*5*. A comparison of these two parameters, however, did not reveal significant differences: The CG content of the two genes is very similar (57% and 60%, respectively), and the choice of most codons is nearly identical.

The knowledge of the *neo* gene structure also allows the comparison of the gene and its protein product to similar enzyme systems that inactivate aminoglycosides by phosphorylation. Cells carrying transposon Tn*5* produce APH type II while transposon Tn*903* codes for type I APH (Jimenez and Davies, 1980). Comparison of the DNA sequence of the corresponding region in Tn*903* (Oka et al., 1981) with Tn*5* DNA revealed an unexpectedly high degree of protein sequence homology between the two apparently different enzymes. At the nucleotide level significant homology was detected in the carboxy terminal parts of the two genes only. However, comparison of the derived amino acid sequences (Fig. 6) shows a rather close relationship between the two enzymes, in that as much as 55% of the amino acid residues are identical or belong to the same functional group. The C-terminal part of the gene products appears to be highly conserved. These results suggest a common origin of the genes of two apparently unrelated enzymes and make a convergent evolution rather unlikely.

The *neo* gene of the Tn*5* appears to be an excellent selection marker for vectors in prokaryotic as well as in eukaryotic systems (Jimenez and Davies, 1980; Colbère-Garapin et al., 1981; Southern and Berg, 1982). The gene is also well suited to screen for cloned DNA fragments by insertional inactivation at five different unique restriction sites inside the coding region (*Pst*I, *Bal*I, *Mst*I, *Pvu*II, and *Sph*I, see Fig. 3). In addition, the short gene element of some 800 bp is flanked by easily accessible restriction sites. Two unique cleavage sites (for *Bgl*II and *Bcl*I) are located some 30 bp ahead of the coding region, and four other sites (*Ava*I, *Sal*I, *Xho*I and *Bam*HI) are not too far from its end (170, 240, 480, and 730 bp, respectively). These sites can be used for oriented cloning of a promoterless *neo* gene in vector constructions. A minimal size gene of 830 bp has been obtained in this work by inserting a new *Hin*dIII site only 17 bp beyond the 3'-end of the gene in mutant pKm244. Finally, heterologous promoter elements can be joined into the *Bgl*II or *Bcl*I site in front of the coding region allowing the expression of APH in many different systems. We have shown for *E. coli* (Reiss, 1982) and *B. subtilis* (Sprengel, 1982) that the levels of antibiotic resistance and of APH synthesis are proportional to the strength of the preceding start signals for gene expression which classifies the *neo* gene also as a good indicator to assess the strength of such regulatory units (Reiss, 1982).

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parable structures were described for genes with mRNAs of high stability, e.g., for outer membrane proteins of *E. coli* (lipoprotein: Nakamura et al., 1980; outer membrane protein II*: Beck and Bremer, 1980).

(b) The *neo* gene and its gene product

The M_r-value of the *neo* gene product synthesized in maxicells as determined by SDS-gel electrophoresis is 27500 (Fig. 4). Similar values were measured for the purified enzyme by gel filtration ($M_r = 27000$; Matsuhashi et al., 1976) or for the protein synthesized in minicells by gel electrophoresis ($M_r = 26000$; Rothstein et al., 1980). As shown in Fig. 3 there is only one open translational reading frame of corresponding length in the DNA sequence starting at the ATG in position 1551 and reaching a TGA stop codon at position 2342. This sequence can code for a protein of 264 amino acid residues with a calculated M_r-value of 29053. This is slightly above the apparent values determined with the protein.

The amino acid composition of the protein calculated from the nucleotide sequence agrees with the data determined with the purified enzyme (Matsuhashi et al., 1976) except for differences in cystine and valine (Table I). The latter cannot be explained by processing of a primary translation product, since both cystine and valine are located in the middle of the protein and not at the C or N terminus, and no evidence for processing could be

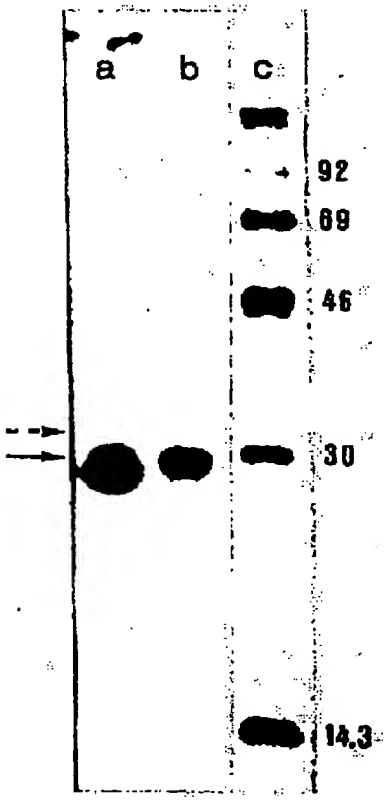

Fig. 4. Expression of APH in maxicells. UV irradiated and starved CSR603 maxicells (Sancar et al., 1979) transformed with pKm2 (a) and pKm22 (b) were incubated for 30 min with ^{35}S-labeled methionine, lysed and the extracts analysed on a 12.5% polyacrylamide-SDS gel. Lane (c) represents a ^{14}C-labeled protein marker. The M_r-values of the proteins are specified on the right margin ($\times 10^3$). The positions of the vector-encoded β-lactamase precursor ($M_r = 31.3$; dashed arrow) and mature protein ($M_r = 28.7$; solid arrow) are indicated.

TABLE I

Amino acid composition of APH

Amino acid	mol %	
	Derived from nucleotide sequence	Determined by amino acid analysis [a]
Ala	13.2	14.7
Arg	7.5	7.5
Asp	9.4	11.4
Asn	1.1	
Cys	1.9	0.0
Glu	6.8	12.0
Gln	4.2	
Gly	7.9	8.3
His	2.6	2.8
Ile	3.8	4.0
Leu	12.1	13.0
Lys	1.5	1.8
Met	2.3	2.3
Phe	4.2	4.4
Pro	4.2	5.2
Ser	3.8	3.5
Thr	3.8	3.6
Trp	1.9	nd [b]
Tyr	1.5	1.9
Val	6.0	3.6

[a] Matsuhashi et al. (1976).
[b] not determined.

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